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BACTERIAL FORMATION OF GLUTAMIC ACID FROM ACETIC ACID

I. GLUTAMATE FORMATION AND ITS RELATED ENZYMES IN BREVIBACTERIUM FLAVUM, No. 2247

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(Received for publication, July 18, 1959)

In the course of our preliminary studies on the glutamate formation from glucose, it was observed that a large amount of α-ketoglutaric acid (αKG*) or glutamic acid (in the presence of ammonium salts) was aerobically formed from acetate as well as from glucose by the cell suspension of a strain of *Brevibacterium flavum*, No. 2247, which had grown on glucose as the sole source of carbon and accumulated L-glutamate in its growing culture medium.

Although evidence was obtained for the presence of aconitase, TPN-linked isocitric dehydrogenase, succinic dehydrogenase, and fumarase in the cell-free extracts of this bacterium (*1*), the Krebs cycle cannot account for the net synthesis of αKG from acetate when this is the sole source of carbon. Recently, the occurrence of a cyclic process, " the glyoxylate cycle ", representing a modification of the Krebs cycle, has been established by Kornberg and Krebs in those organisms, which can derive all their carbon requirements from two-carbon compounds, such as acetate or ethanol (*2*). From this point of view, the occurrence in this bacterium (*1*) of isocitritase (*3–5*), which catalyses the aldol fission of isocitrate to succinate and glyoxylate, suggests that the accumulation of α-ketoglutarate involves the glyoxylate cycle.

The present paper reports some results on the formation of glutamic acid and αKG by an intact cell suspension and the enzymatic reactions involving acetate or its activated form in cell-free extracts of this bacterium. From these results, a possible pathway of glutamate formation was proposed.

EXPERIMENTAL

Washed Cell Suspension and Cell-free Extract—After *Brevibacterium flavum* No. 2247 was grown aerobically in a medium described previously containing glucose and urea as a carbon and nitrogen source, respectively, at 30° for 24 hours, washed cell suspension and cell-free extract were prepared as described previously (*1*).

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* The abbreviations used throughout this paper include αKG, α-ketoglutaric acid; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; DiNPH, 2,4-dinitrophenylhydrazone or 2,4-dinitrophenylhydrazine; ΔE_{340}, increment of optical density at 340 mμ (light path: 1 cm); ATP, adenosine triphosphate; GSH, reduced glutathione; CoA, coenzyme A; cpm, counts per minute; Tris, tris (hydroxymethyl)aminomethane.

Chemicals—Glyoxylic acid, L-malic acid, oxaloacetic acid, ATP, CoA, and pyridine nucleotides were purchased from the California Corporation for Biochemical Research, and αKG from the Nutritional Biochemicals Corporation. Sodium acetate-2-C^{14} and $BaC^{14}O_3$ were obtained from the Daiichi Pure Chemicals Co., Ltd. and the Radiochemical Centre, respectively. Sodium carbonate-C^{14} solution in 0.01 N NaOH was prepared from about 0.3 mc. of $BaC^{14}O_3$ by liberation of $C^{14}O_2$ with 2 ml. of 1 per cent perchloric acid in an evacuated system and introducing the $C^{14}O_2$ in 5 ml. of 0.01 N NaOH.

Chemical Determination—Pyruvate and αKG were measured as DiNPH by the procedure of Friedemann and Haugen (*6*, *7*), and glutamic acid was determined by the manometric method (*8*) using acetone dried cells of *E. coli* "Crookes" strain, a specific L-glutamic decarboxylase preparation. Oxygen uptake and carbon dioxide evolution were measured by the usual manometric methods. Methods for the determination of nitrogen content of cell materials and for paperchromatography of amino acids and keto acids were described in our previous paper (*1*, *9*).

Demonstration and Measurement of Enzyme Activities—Malic dehydrogenase was demonstrated spectrophotometrically by a modification of the procedure of Ochoa (*10*), or of Straub (*11*). Acetate activating enzyme was determined by the method of Jones and Lipmann (*12*), which was based on the fact that activated acetate reacted rapidly with hydroxylamine at neutral pH to form hydroxamic acid. A hydroxamic acid standard solution was prepared from succinic anhydride (*13*).

Paper-chromatography of Hydroxamic Acid—After reactions were stopped by the addition of nine-fold volume of ethanol, the reaction mixtures were centrifuged and the supernatant solutions were concentrated on a water bath at 60~70°. Then, the concentrated solutions were dried in a vacuum desiccator at room temperature, the residues were extracted with a small volume of ethanol, and the ethanol extracts were chromatographed in *n*-butanol saturated with water (*14*) or in phenol-water (10 : 2 v/v) after being spotted on Toyo No. 51 paper. The resulting hydroxamic acid spot was detected by spraying with a solution of ferric chloride (50 g. of $FeCl_3 . 6 H_2O$ in 1 liter of 95% ethanol containing 0.1 mole of HCl) (*14*). Acetohydroxamic and succinomonohydroxamic acid were prepared by mixing 0.1 g. of acetic or succinic anhydride, respectively, with 4 ml. of 2 M neutralized aqueous solution of hydroxylamine prepared freshly, and then by the same treatment as described above.

Incorporation of C^{14} from Acetate-2-C^{14} (*15*, *16*)—The incubation mixture used contained 100 μM of potassium phosphate, pH 7.5, 10 μM of $MgCl_2$, 0.08 μM of CoA, 10 μM of ATP, 10 μM of acetate-2-C^{14} (giving 6.42×10^5 cpm.), 0.25 ml. of bacterial extract and water in a total volume of 0.9 ml. The mixture was incubated at 30° for 20 minutes, after which time were added 10 μM of unlabelled materials (0.1 ml.) as indicated. Incubations were continued for various lengths of time, the reactions being stopped by the addition of 3 ml. of hot absolute ethanol. Methods for paperchromatographic separation, determination, and identification of labelled materials formed were essentially the same as that of Kornberg *et al.* (*15*, *16*). Paperchromatographic solvents used were phenol-formic acid (500 g- of phenol : 13 ml. of 90 per cent HCOOH : 167 ml. of water), *n*-butanol-propionic acid-water (10 : 5 : 7), and *n*-butanol- formic acid-water (4 : 1.5 : 1) (*15*, *16*, *17*).

Incorporation of C^{14} from C^{14}-Carbon Dioxide—The experiments on the incorporation of C^{14} from C^{14}-carbon dioxide were performed in standard Warburg vessels in air at 37°. The reaction mixture used contained 50 μM of phosphate buffer, pH 7.5, 10 μM of ammonium carbonate, 30 μM of unlabelled sodium acetate, 10^6 cpm. of C^{14}- sodium carbonate (in 0.01 *N* NaOH), and 0.54 mg. N of washed cell suspension, and was made up with water to 1.6 ml. After 5 hours of aerobic incubation, the reaction was stopped by addition of 0.5 ml. of 0.5 *N* HCl, carbon dioxide was removed by aeration, and then the reaction mixture was centrifuged. Glutamic acid formed was separated by two-dimensional paperchromatography (solvents: *n*-butanol—acetic acid—water (4 : 1 : 1), and phenol—water (10 : 2)) and its specific radioactivity was measured as described in our previous paper (*9*). The specific radioactivity of carbon-2 to -5 in the labelled glutamic acid molecule was also measured after the conversion to γ-aminobutyric acid by glutamic decarboxylase of dried *E. coli* (Crookes strain) cells as described previously (*9*).

RESULTS

α-Ketoglutarate Formation as an End Product in Acetate Oxidation by Resting Cells—Complete oxidation of one mole of acetate involves the following reaction:

$$C_2H_4O_2 + 2O_2 = 2CO_2 + 2H_2O$$

Although *Brevibacterium flavum* No. 2247 oxidized acetate rapidly, oxygen absorption per mole stopped at the considerably lower level than indicated by the above equation. A typical example was:

$$C_2H_4O_2 + 1.33\ O_2 \longrightarrow 1.18\ CO_2 + 0.10\ C_5H_6O_5$$

The carbonyl compound formed as an end product in this reaction was identified as αKG by paperchromatography (solvent: *n*-butanol—ethanol—0.5 *N* NH_4OH, 7 : 1 : 2), the characteristic absorption spectrum (Fig. 1.), and melting point of its DiNPH, which was recrystallized from water.

	Melting point (uncorrected)
Standard αKG-DiNPH	211.4°
End product-DiNPH	209.9°
Mixture of above two DiNPH	210.8°

L-Glutamate Formation from Acetate and Incorporation of CO_2 by Resting Cells—As shown in Fig. 2, a large amount of L-glutamate was formed aerobically from acetate and ammonium ions by a resting cell suspension. The amount of glutamate accumulated varied from 0.13 to 0.21 moles per mole of acetate with the conditions of the reaction and with the preparations of resting cells. A small amount of αKG was also measured. Alanine and pyruvate, which had been found as reaction products in the oxidation of glucose (*6*), were not detected in this case.

Under similar conditions of the reaction, the incorporation of C^{14} from C^{14}-carbon dioxide to glutamate formed from acetate was demonstrated by radioautogram of the reaction products.

The specific radioactivity of glutamate formed was 3,510 cpm per μM, and ninety five per cent of C^{14} incorporated in the glutamate molecules were detected in α-carboxyl carbon.

Reactions of Acetate—Cell-free extracts of *Brevibacterium flavum* No. 2247 did not consume oxygen when acetate was added. Enzymic assay by the method of Jones and Lipmann (*12*) showed, however, that such extracts were still capable of activating acetate, in the presence of ATP, to form hydroxamic acid (Fig. 3), which was identified by paperchromatography as acetohydroxamic acid and not as succinomonohydroxamic acid. Although the reaction did not require coenzyme A, the data in Fig. 3 can not be regarded as definite evidence for the presence of acetokinase (*18*) in so far as aceto-CoA-kinase (*12*) will give rise to acetohydroxamic acid in the presence of catalytic concentrations of CoA. For ruling out the presence of the latter enzyme, effects of CoA on this reaction were examined by coupling the reaction with an acetyl acceptor enzyme system such as condensing enzyme (*19*) or malate synthetase (*20*), which required catalytic amounts of CoA. (Table I). The data show that the main path of acetyl CoA formation may involve the reactions catalysed successively by aceto-kinase and phosphotransacetylase (*21*). Reactions of acetate-2-C^{14} with tricarboxylic acid-cycle intermediates by the cell-free extract were examined in the presence of added CoA and ATP as shown in Table II. The presence of condensing enzyme was show by the heavy incorporation of C^{14} into citrate on addition of oxaloacetate (Table II, Fig. 4). Considerable amounts of C^{14} appeared also in malate. Since it was known that this bacterium contained isocitritase and aconitase (*1*), this finding suggested that malate might be formed in this extract via the actions of aconitase, isocitritase and malate synthetase. That malate synthetase was present in the extract was shown by the heavy incorporation of C^{14} from acetate into malate in the presence of glyoxylate (Table II, Fig. 5). The combined action of aconitase, isocitritase, and malate synthetase was demonstrated by the similar formation of labelled malate from labelled acetate and citrate (Table II). Amongst the substrates tested,

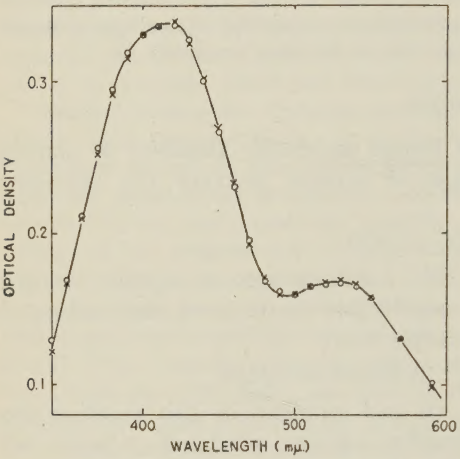


FIG. 1. Absorption spectra of 2,4-dinitrophenylhydrazones of standard α-ketoglutarate (—○—) and carbonyl compound formed by oxidation of acetate (—×—) in 1 *N* sodium hydroxide.

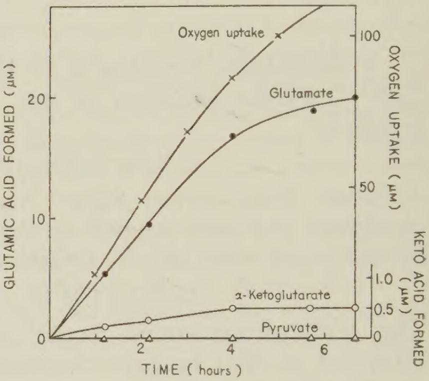


FIG. 2. Glutamate formation from acetate and ammonium ions. Reaction mixture contained 250 μM of phosphate buffer, pH 7.0, 75 μM of sodium acetate, 75 μM of ammonium acetate, 0.88 mg. N of washed cell suspension, and water in a total volume of 2.5 ml. in a Warburg vessel. Incubated at 37° with shaking.

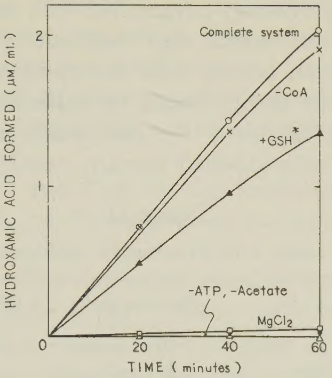


FIG. 3. Hydroxamic acid formation from acetate and hydroxylamine. Complete system contained 100 μM of potassium phosphate, pH 7.5, 10 μM of $MgCl_2$, 50 μM of KF, 10 μM of ATP, 0.08 μM of CoA, 20 μM of potassium acetate, 200 μM of freshly neutralized hydroxylamine, pH 7.4, 0.12 mg.N of cell-free dialysed extract, and water in a total volume of 1 ml. at 30°.

* 10 μM of reduced glutathione was added.

TABLE I

Effect of Coenzyme A on the Reactions of Acetate with Hydroxylamine, Oxaloacetate, and Glyoxylate

Enzyme[4]	CoA	Hydroxamic acid formed[1] μM/ml.	Citrate formed[2] cpm/ml.	Malate formed[3] cpm/ml.
Dialysed extract	+	1.26	13,320	4,000
,,	—	1.21	505	302
Resin-treated extract	+	0.80	3,660	1,598
,,	—	0.79	181	208

1) Conditions were essentially the same as in Fig. 3. Incubation was at 30° for 30 minutes.

2) and 3). Reaction mixture contained 100 μM of potassium phosphate, pH 7.5, 10 μM of $MgCl_2$, 10 μM of ATP, 0.08 μM of CoA, 0.25 ml. of cell-free extract, 10 μM of oxaloacetate or glyoxylate (for 2) and 3), respectively), 10 μM of acetate-2-C^{14} (containing 6.42×10^5 cpm of C^{14}), and water to make up the total volume to 1 ml. Incubations were at 30° for 40 and 60 minutes, respectively.

4) Dialysed extract (0.478 mg.N/ml.): Cell-free extract was dialysed aginst 0.01 *M* Tris buffer, pH 7.5, for 2 hours at 4°. Resin-treated extract (0.309 mg.N/ml.): 10 ml. of cell-free extract were treated with 10 g. of Dowex 1-Cl (wet, 200~400 mesh) for 30 minutes at 0~2°, pH 7.5~8.0.

only glyoxylate and its precursor, citrate, led to a major incorporation of C^{14} into malate. Succinate and αKG were without effect. In the presence of unlabelled malate, C^{14} from acetate was incorporated chiefly into citrate or isocitrate (Table II). This finding could be explained by the formation from malate of oxaloacetate, catalysed by the action of malic dehydrogenase, which, by condensation with labelled acetate, would lead to the formation

Table II

Reactions of Acetate with Tricarboxylic Acid-Cycle Intermediates

Compound added	Radioactivity ($\times 10^2$ cpm) in			
	Malate	Fumarate	Succinate	Citrate or isocitrate
None	0.56	—	2.20	0.96
Glyoxylate	58.4	13.65	4.45	—
Succinate	—	—	1.44	—
α-ketoglutarate	0.75	—	1.83	1.17
Citrate	20.9	5.45	2.03	2.64
Oxaloacetate	16.2	1.46	3.06	228.0
Malate	1.22	—	—	24.0

— : Corresponding spot was not detected.

Reaction mixture contained 100 μM of potassium phosphate, pH 7.5, 10 μM of $MgCl_2$, 0.08 μM of coenzyme A, 10 μM of ATP, 10 μM acetate-2-C^{14} (containing 6.42×10^5 cpm of C^{14}), 0.19 mg. N of cell-free extract (dialysed for 2 hrs.), and water to make up to the total volume to 0.9 ml. After preincubation of 20 minutes at 30°, 10 μM of unlabelled substrates (0.1 ml.) as indicated were added and incubation was contained for 1 hour at 30°.

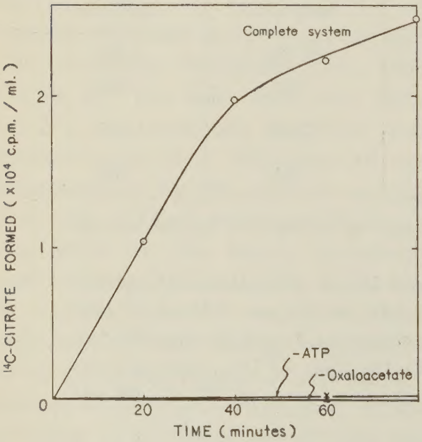


FIG. 4. C^{14}-Citrate formation from acetate-2-C^{14} and Oxaloacetate. Condition were the same as given in Table II.

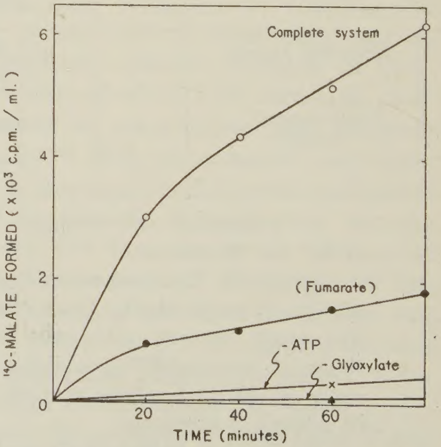


FIG. 5. C^{14}-Malate (and C^{14}-fumarate) formation from acetate-2-C^{14} and glyoxylate. Conditions were the same as given in Table II.

of labelled citrate. The formation of oxaloacetate from malate in this sequence of reactions would require a concomitant reduction of another substrate, the nature of which is not known.

That the formation of labelled malate was accompanied by the formation of labelled fumarate could be explained by the presence of fumarase in the extract (*1*).

Requirement of ATP and CoA for the formation of citrate and malate from acetate (Table I, Fig. 4, 5) suggests an intermediary formation of acetyl CoA in these reactions.

Reactions of Malate—The bacterial extracts contained both malic dehydrogenase and malic enzyme (*22*). Malic dehydrogenase was assayed by the oxidation of DPNH caused by the addition of oxaloacetate (Table III). This reaction could not be explained by the combined action of oxaloacetic carboxylase (*1*) and lactic dehydrogenase or malic enzyme, since pyruvate did not oxidize DPNH in the presence of the extracts and bicarbonate. Malic dehydrogenase was also demonstrated by the reduction of DPN in the

TABLE III

Demonstration of Malic Dehydrogenase and Malic Enzyme and Their Properties

Enzyme related	Substrate	Reaction mixture and condition	Reaction rate
Malic dehydrogenase	Oxaloacetate	(R-I), 25°	2.1×10^1 $(-\Delta E_{340})$/min.
	,,	(R-I)+Mn^{++}, ,,	1.9 ,,
	,,	(R-I)−DPNH, ,,	0.0 ,,
	,,	(R-I)−extract, ,,	0.0 ,,
	,,	(R-I)−substrate, ,,	0.2 ,,
	Pyruvate	(R-I)+HCO_3^-, ,,	0.2 ,,
Malic dehydrogenase	L-Malate	(R-II), DPN, 25°	2.1×10^{-2} ΔE_{340}/min.
	,,	(R-II), TPN, ,,	0.25 ,,
	,,	(R-II)−substrate, DPN, ,,	1.2 ,,
	,,	(R-II)−substrate, TPN, ,,	0.45 ,,
	,,	(R-II)+Mn^{++}, DPN, ,,	2.4 ,,
Malic enzyme	L-Malate	(R-III), TPN, ,,	0.4 ,,
	,,	(R-III)+Mn^{++}, TPN, ,,	0.6 ,,
	,,	(R-III), DPN, ,,	0.04 ,,
	,,	(R-III)+Mn^{++}, DPN, ,,	0.04 ,,
	,,	(R-III)+substrate, TPN, ,,	0.01 ,,

Reaction mixtures contained the following reactants in micromoles per ml.

(R-I): Tris buffer, pH 7.5, 50; substrate, 0.25; DPNH, 0.075; and cell-free extract (dialysed for 2.5 hrs.), 0.031 mg.N;.

(R-II): Tris buffer, pH 7.5, 50; sodium L-malate, 10; DPN or TPN, 0.125; KCN, 100; and cell-free extract (dialysed for 2 hrs), 0.032 mgN. The amount of $MnSO_4$ and bicarbonate added was 0.5 and 0.25 μM per ml.

(R-III): (R-II)−KCN.

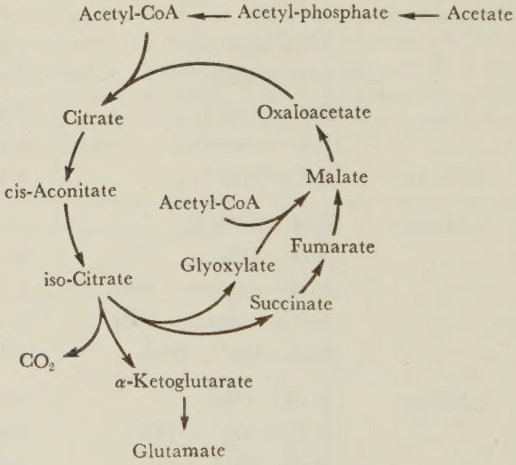
presence of L-malate and cyanide to bind the oxaloacetate formed. The data show that this enzyme is DPN-specific and does not require manganese ions (Table III). In the absence of cyanide, reduction of DPN did not occur but TPN reduction was observed and required manganese ions for maximum activity, indicating the presence of TPN-linked malic enzyme (Table III) (*1*).

DISCUSSION

When α-ketoglutarate is produced from complex substrates, carbon dioxide-fixing reactions, such as form oxaloacetate or malate from pyruvate, are thought to replenish the supply of tricarboxylic acid cycle intermediates. Such reactions are not, however, sufficient to explain the processes by which αKG can be produced from simple substrates, such as ethanol or acetate, as sole carbon sources. The tricarboxylic acid cycle is a means whereby acetate, after reacting with oxaloacetate, is oxidized to carbon dioxide, with the regeneration of oxaloacetate. Any removal of intermediates from the cycle would prevent the regeneration of oxaloacetate, and would therefore cause the cycle to stop. Mechanisms must therefore operate under these conditions whereby the acceptor for C_2 units can be synthesized from acetate.

SCHEME I.

Probable Pathway of Glutamate Formation from Acetate



The experiments reported in this paper provide evidence that acetyl-coenzyme A can react both with oxaloacetate to form citrate and with glyoxylate to form malate. The aldol fission of isocitrate provides the glyoxylate necessary for this latter condensation (*1*). Since the extracts also contain the enzymes capable of catalysing the conversion of citrate into isocitrate, of malate into oxaloacetate, of succinate into malate, and of isocitrate into αKG (*1*), it is possible to construct a modification of the tricarboxylic acid cycle leading to the net synthesis of αKG from acetate (Scheme I).

A direct condensation of two molecules of acetate to give succinate, first postulated by Thunberg (*23*), would similarly supply necessary C_4 compounds. There has been no evidence for the occurrence of this reaction. Although Seaman and Naschke (*24*) have recently claimed a direct formation of succinate from acetyl-CoA to occur in *Tetrahymena* and *E. coli*, no evidence for this reaction was obtained with cell-free extracts of *Brevibacterium flavum.*

The scheme I can also explain the incorporation of C^{14} of CO_2 to α-carboxyl carbon of glutamate formed from acetate, provided that isotope-exchange reactions are present relating C_4 compounds with C_3 units and carbon dioxide. The latter may be partially supported by the demonstration of malic enzyme in the bacterial extracts.

Scheme I indicates that the reaction of αKG formation from isocitrate is the only one requiring TPN as a coenzyme in the pathway of αKG formation from acetate and that reduced TPN must therefore be reoxidized by oxygen through a terminal respiratory enzyme system or an oxidase. In our previous paper (*6*), it was reported that αKG and glutamate were not oxidized nor decarboxylated but transaminated by intact cells and that glutamic dehydrogenase activity of cell extracts was considerably high. These results suggest that the inability of cells for the oxidation of glutamate must be ascribed to an inactive state of glutamic dehydrogenase in intact cells. Therefore, the reaction of glutamate formation from αKG may not be catalysed by glutamic dehydrogenase in this bacterium.

SUMMARY

1. α-Ketoglutarate or glutamate (in the presence of ammonium ions) was formed aerobically from acetate by resting cells of *Brevibacterium flavum*, No. 2247.

2. When C^{14}-carbon dioxide was added to the latter reaction system, glutamate formed was labelled almost exclusively in the α-carboxyl carbon.

3. Reactions of acetate were studied in cell-free extracts of this bacterium. Such extracts are capable of catalysing the formation of acetyl-CoA from acetate, presumably via acetyl-phosphate.

4. Acetyl-CoA thus formed reacts both with oxaloacetate, to form citrate, and with glyoxylate to form malate.

5. Evidence for the presence of DPN-specific malic dehydrogenase and TPN-specific malic enzyme was obtained with cell-free extracts.

6. From these results, a probable pathway of glutamate formation from acetate was proposed.

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The Journal of Biochemistry, Vol. 47, No. 3, 1960

STUDIES ON ENZYMATIC SYNTHESIS OF COCARBOXYLASE IN ANIMAL TISSUE

III. PURIFICATION AND PROPERTIES OF THIAMINOKINASE FROM RAT LIVER*

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A representative method hitherto used extensively for the preparation of thiaminokinase from animal tissue is that of Leuthardt and Nielsen (*1*). Reports on other methods have been presented by Weil-Malherbe (*2*) and Steyn-Parvé (*3*) on the purification of thiaminokinase from baker's yeast. However, these preparations are not suitable for detailed studies on the reaction mechanism, because of marked contamination with other interfering enzymes and of low yield. The present paper describes the purification and some properties of the enzyme.

EXPERIMENTAL AND RESULTS

Assay for the activity of the enzyme was made by the method described previously (*4*) with the phosphate-free system except the alteration of pH of the reaction mixture to 8.0. The materials used for the determination were all of the similar grade to those of the previous communications (*4*, *5*). The activities of adenosinetriphosphatase and adenylate kinase were determined by a method similar to that of a previous paper (*6*). For the measurement of the protein content, spectrophotometric method according to Warburg and Christian (*7*) was applied. Alumina C_γ-gel was prepared by Willstätter's method (*8*) and the preparation used for the experiment was aged for about one year. Electrophoresis was made by using Tiselius' apparatus (*9*) and agar electrophoresis was carried out by the method of Giri (*10*). Semiquantitative measurement of protein on the agar plate was made by the use of a densitometer after staining with Amidoschwartz.

Preparation of the Enzyme

After a number of trials for the purification of thiaminokinase, the method described below was found to be the most commendable, and is composed of the following six steps:

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* The following abbreviations are used in this paper: TDP, thiamine diphosphate (cocarboxylase); Tris, tris(hydroxymethyl)aminomethane; ATPase, adenosinetriphosphatase.

Step 1. Preparation of Acetone Dried Powder: Rat liver obtained under anaethesia by ether was thoroughly perfused with cold physiological saline, and about ten volumes of ice-cold acetone was added to the minced liver after dehydration with filter paper. The tissue was homogenized twice with Waring blendor under ice-chilling. Resultant homogenate was spun down, and the packed precipitate was homogenized again as above. After the second centrifugation, homogenization was repeated twice more, packing to a cake under filtration with suction instead of the centrifugation described above. Thus resultant cake was crumbled and powdered rapidly in a mortar. The acetone-dried powder could be stored in a desiccator at a low temperature without a marked loss of activity for about three months.

Step 2. Extraction: The acetone dried-powder so obtained was extracted with ten volumes of 0.05 *M* Tris buffer of pH 7.8 by the use of Potter-Elvehjem type homogenizer. At first, the powder was ground with addition of six volumes of the buffer, then the packed precipitate after centrifugation was extracted again with four volumes of the buffer, each supernatant was combined and subjected to the next step. The extract was an opalescent orange solution.

Step 3. Heat-Treatment: The pH of the original extract was adjusted to 5.8 with cautious addition of 0.5 *N* acetic acid under continuous stirring. The turbid solution was warmed in a water bath to 52° for 5 minutes with vigorous stirring. The enzyme solution was cooled in an ice bath immediately after the treatment and the precipitate formed was spun down and discarded. The solution thereby obtained was clear orange.

Step 4. Ammonium Sulfate Fractionation: To the supernatant of the heat-treatment, solid ammonium sulfate, which was pulverized in advance, was added in small portions to 0.52 saturation (32.8 g. for every 100 ml. of the extract) under mechanical stirring. After standing for about five hours the precipitate formed was spun down and discarded. To the supernatant another portion of solid ammonium sulfate was added to 0.68 saturation (additional 10.3 g. for every 100 ml of the resulting solution) as above and the mixture was kept overnight in an ice box. The precipitate obtained was dissolved in a small amount of water and dialyzed against distilled water for at least six hours at 0°. The insolved matter here formed was removed by centrifugation, and the solution was made an appropriate volume by addition of water so as to bring the protein concentration to under 1 per cent.

Step 5. Acid Treatment: The pH of the above solution was adjusted to 4.3 by a slow addition of 0.5 *N* acetic acid, and 0.02 volume of 0.5 *M* acetate buffer of pH 4.3 was added. The solution was incubated at 37° for 30 minutes under occasional stirring. Strongly flocculated precipitate was centrifuged off and the light yellow supernatant was subjected immediately to the next step.

Step 6. Alumina Cγ-gel Chromatography: Enzyme solution obtained by the former step was passed under constant pressure through a gel column which was composed of alumina Cγ-gel and Hyflo Super-cel mixed in a ratio of 1 : 5 by weight. Amount of the gel used was in a ratio of 2 : 1 to the protein content and the column was bufferized scfficiently with 0.01 *M* acetate buffer of pH 4.3 before use. After charge of the enzyme the column was washed thoroughly with 0.1 *M* KH_2PO_4 until protein in the eluate disappeared. Then the elution system was changed to the gradient one according to the original method of Hurlbert *et al.* (*12*), under constant pressure. At first, 0.133 *M* phosphate buffer of pH 6.7 was used as a reservoir system against about 100 ml. of 0.1 *M* KH_2PO_4 of a mixer system. When the pH of the eluate reached 5.2, the reservoir system was changed again to 0.16 *M* Na_2HPO_4 as illustrated in Fig. 1. The fraction eluted between pH 5.2 and 5.8 was collected and ammonium sulfate was added to saturation. After standing overnight the precipitate was spun down by a high-speed centrifuge, the protein pellet was dissolved in water, and used for the experiment after dialysis against water.

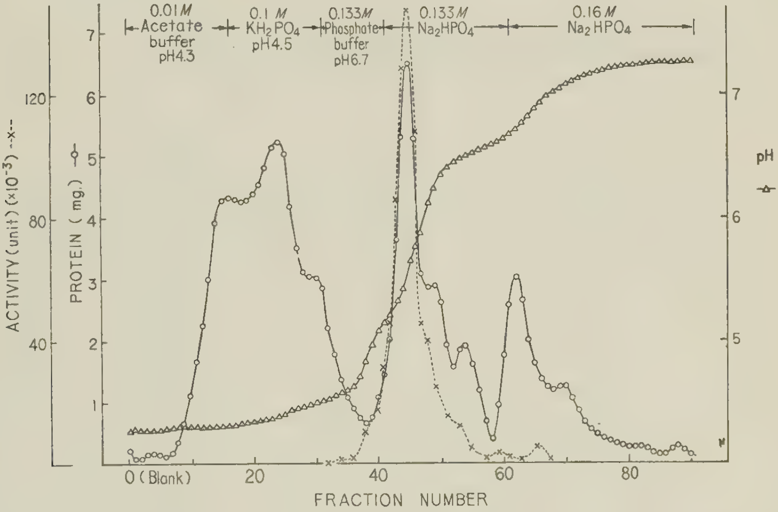


FIG. 1. Separation of the enzyme by alumina C$_\gamma$-gel chromatography. The enzyme obtained from Step 5, weighing 105 mg. protein, was placed on the column (4 cm × 3.5 cm) and eluted by gradient system (*12*) with appropriate change of reservoir system as illustrated under constant pressure at 0°–2°. The elution velocity was maintained at 0.8 ml./10 min. throughout the experiment. ---×---, Activity; —○—, protein; —△—, pH.

Process of the purification is summarized in Table I.

Properties of the Enzyme

The enzyme was present mainly in the fraction between 0.52 and 0.68 saturation by salting out with ammonium sulfate (Fig. 2) and it may belong to an albumin-type protein. Some organic solvents could be used to fractionate

TABLE I

Summary of Purification Procedure

Fraction	Total volume ml.	Total units thousand	Protein mg.	Specific activity units/mg[1]	Recovery %
Step 1	(50 g)	—	—	—	—
Step 2	405	2410	13940	186	100
Step 3	390	2239[2]	2845	786	93[2]
Step 4	50	1062	350	3182	44
Step 5	50	772	105	7346	32
Step 6	5	258	11.5	22427	10.7

1) 1 unit: 1 mµg of TDP formed under standard condition (*4*).

2) Increase of total activity was not observed in this case.

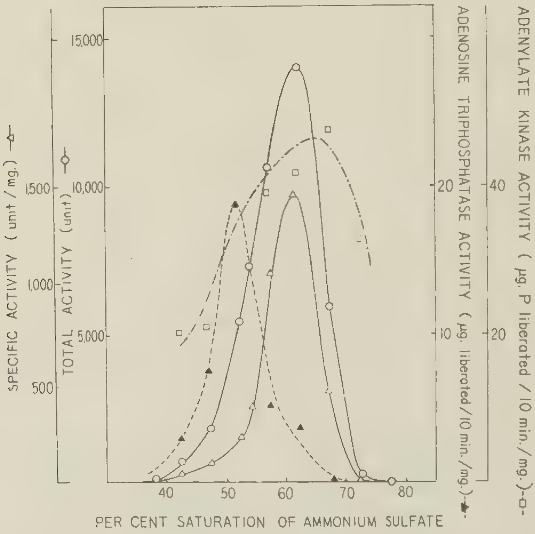


FIG. 2. Distribution of thiaminokinase and some related enzymes on ammonium sulfate fractionation. Activity value was plotted at the point of mean ammonium sulfate saturation of each fraction. —△—, Specific activity; —○—, Total activity; ---▽---, Adenosinetriphosphatase (ATPase) activity; ---□---, Adenylate kinase activity.

the enzyme from other indifferent proteins but it was accompanied with a considerable denaturation even at a low temperature. The main fraction was precipitated with 52 to 62 per cent methanol or with 36 to 46 per cent acetone. All efforts to separate the enzyme with ion exchangers, diethylaminoethyl-, phospho-, and carboxymethyl-cellulose, resulted equally in a disappearance of a greater part of the activity.

Stability of the enzyme was markedly affected by its pH as indicated in Fig. 3, which shows a constant maximal stability in a region of pH 7.2 to 5.8. The same relationship was found to be held in a frozen state under

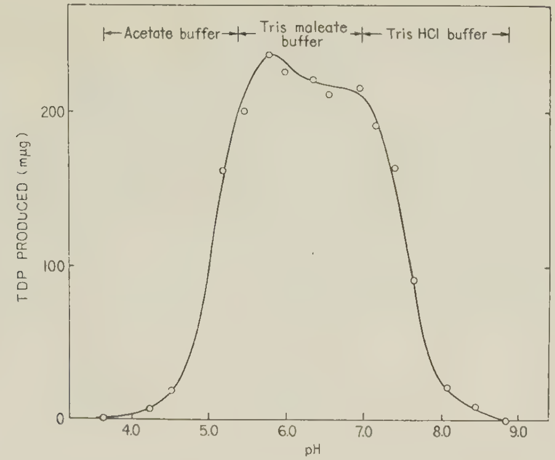
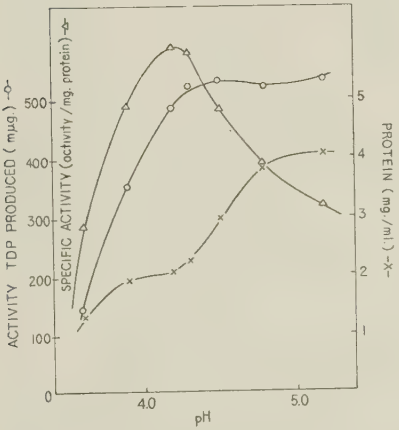


FIG. 3. Stability of the enzyme at various pH. Same aliquots of the enzyme (3.5 mg.) corresponding to Step 4 were preincubated with each buffer at 37° for 5 hours. After the treatment, pH of the tubes was adjusted to 8.0, filling up to a constant volume, and the activity was assayed as before.



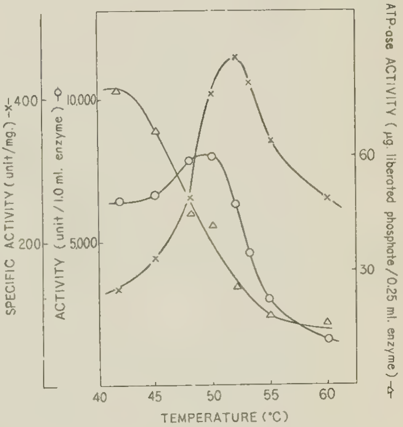


FIG. 4. Effect of acid treatment. A definite portion of the enzyme was preincubated in 0.05 *M* acetate buffer at various pHs at 37° for 30 minutes. The solution obtained after removal of insoluble matter formed by centrifugation, the pH was adjusted to 8.0 followed by the addition of water to a constant volume, and an aliquot of the solution was subjected to determination of the activity. —○—, Activity remained in the supernatant; —△—, Specific activity; —×—, Protein remained in the supernatant.

FIG. 5. Effect of heat treatment. The enzyme from Step 2 was heated at pH 5.8 for 5 minutes at the specified temperature. Adjustment of pH to that of each assay system was followed by determination of the activities. —×—, Specific activity; —○—, Total activity; —△—, ATPase activity.

storage, and these facts should be kept in mind in calculation of specific activity in comparing each step of purification. The enzyme maintained its activity for a shorter period up to more acidic pH 4.3 under the condition studied, and after exposure of the enzyme to this acidity of pH 4.3, the specific activity became the highest as shown in Fig. 4. Moreover, the enzyme was resistant even to more drastic condition of pH 1.0 at −5° for ten minutes leaving about 65 per cent of the original activity. On the other hand, the enzyme showed a considerable stability to heat, and withstood heating at 52° for 5 minutes, preserving its complete activity. The effect of heating on the total activity was not affected by pH set between 7.2 and 5.8 giving maximal specific activity at the latter. A peculier phenomenon of increasing total activity from 45° to 52° was disclosed to originate from selective denaturation of ATPase which contaminates the preparation (Fig. 5). The enzyme derived from Step 6 showed no coagulation on heating at 100° for one minute. For preservation of the activity for a long time the preparation may be stored in a frozen and concentrated state. In this way the activity remained for about three months without serious loss. Treatment of lyophilization brought about a great loss of activity as a result of denaturation.

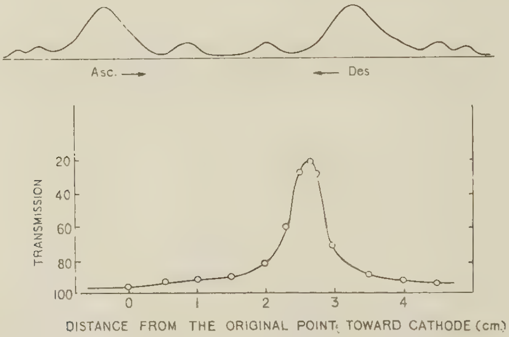


FIG. 6. Electrophoretic patterns of the preparation from Step 6. Upper graph : Schlieren-diagonal pattern obtained after electrophoresis for 30,000 sec. at 9 mA/cm in phosphate buffer of pH 7.25 ; ionic strength, 0.05 ; protein concentration, 0.8 per cent ; bar angle, 60°. Lower graph : Densitometric pattern by staining with Amidoschwartz after zone-electrophoresis on agar plate (25 × 12 × 0.2 mm) for 3 hours at 7 v/cm, 5 mA/cm^2 in veronal buffer of pH 7.40 ; ionic strength, 0.05.

The preparation obtained in the earlier steps contained some enzyme activities which may interfere in the main reaction (cf. Fig. 2). Nucleoside diphosphokinase and ATPase were removed at Step 3 and 5, respectively, and the most troublesome contaminant, adenylate kinase, was eliminated mostly at the last step. The enzyme which decomposes TDP was not contained even in the original extract and the behaviour of the enzymes which affect various derivatives of thiamine except its phosphate will be referred in the next paper.

The purity of the preparation was checked at the last two steps by electrophoretic analysis. The preparation corresponding to Step 5 showed seven peaks in electrophoretic study on agar, whereas that of the last step showed only a single peak at pH 8.6 and 7.4, and yet three peaks was detected in the latter preparation with the Tiselius' apparatus at pH 7.25 and 6.75, showing 68 per cent purity by a rough estimation (Fig. 6) assuming the main peak to be that of the enzyme since the increase of its content was parallel to the specific activity. Attempts for separation of these fractions by means of electrophoretic fractionation were all in vain.

Detailed studies on the action of this purified enzyme will be reported in the near future.

SUMMARY

Thiaminokinase from rat liver was purified about 120 fold compared with the extract from acetone-dried powder and the method for its preparation was described. The purification consisted of the following six steps: 1) Preparation of acetone-dried powder, 2) extraction with 0.05 M Tris buffer of pH 7.8, 3) heat treatment at 52° for 5 minutes at pH 5.8, 4) ammonium sulfate fractionation (0.52 to 0.68 saturation), 5) acid treatment at pH 4.3 for 30 minutes at 37°, and 6) alumina C_γ-gel column chromatography with phosphate buffer of various concentrations as a elution system. The enzyme was stable between pH 7.2 and 5.8, and showed a considerable resistance to acid treatment. The preparation derived from the last step showed about 68 per cent purity by electrophoresis and was practically free from the activities of adenylate kinase, ATPase, nucleoside diphosphokinase, and thiamine pyrophosphatase. Some descriptions on its properties were also made in brief.

The author's thanks are due to Prof. N. Shimazono for his guidance and encouragement during the course of this work.

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CYTOCHROMES OF PSEUDOMONAS-P

THEIR FORMATION AND LOCALIZATION ON CELLULAR COMPONENTS*

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During the course of the investigation on the adaptive formation of pyrocatechase by *Pseudomonas-P*, one of the authors (H. M.) (*1*) has observed the color change of the harvested cells from white to pink as the cell growth proceded. It was confirmed in the present investigation that the color change was mainly due to the cytochrome content. Accordingly this microorganism was used for the study of cytochrome formation.

Up to the present time, there have been many reports (*2–14*) about the variation of bacterial cytochromes in response to the cultivation conditions, for example, the change of oxygen tension or metal deficiency in the growth medium. However, there have been less extensive observations about the variation of cytochrome content of microorganism in response to the growth phase.

Recently, Shibata devised the opal glass method (*15*), which facilitates the spectrophotometry of bacterial cell suspension. Horio succeeded in the purification of some cytochrome components from *Pseudomonas aeruginosa* (*16*). Minakami (one of the authors) investigated heme synthesis in mammalian tissue preparations making use of radioactive iron (*17*). The present investigation was carried out, based on these advances in experimental methods.

MATERIALS AND METHODS

Microorganism—Pseudomonas-P, which was previously isolated and identified to be a strain of Pseudomonas (*18*), was cultivated at 30° under aerobic condition in the following two media: 1. Bouillon-peptone medium containing 1 per cent meat extract, 1 per cent peptone and 0.2 per cent sodium chloride in 1 : 1 mixture of city water and distilled water, adjusted to pH 7.0 with 10 per cent dipotassium hydrogen phosphate. 2. Casamic acid-glucose medium containing 8 volumes of A and 1 volume of B. A contains

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* This investigation has been presented at the 10th Symposium on Enzyme Chemistry, July 1958, Sapporo.

90 mg. of vitamin-free casamic acid (Difco Lac. Inc.) and 300 mg. of potassium dihydrogen phosphate in 50 ml. of distilled water, adjusted to pH 7.0 with *N* potassium hydroxide. B contains 500 mg. of glucose and 8 mg. of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) in 20 ml. of distilled water. A and B were separately sterilized and mixed just before use.

The cultivation was carried out in 500 ml. Sakaguchi flask containing 150 ml. of the medium or in 25 ml. Monod-type test tube containing 10 ml. of the medium. The cell suspension, previously cultivated for 15 hours under the same conditions, was inoculated to the new medium in a concentration of 1 per cent. The cells were harvested by centrifugation at appropriate time interval, washed several times with cold 0.2 per cent potassium chloride and once with distilled water. The cell growth was estimated by a turbidimetric method or by measuring the dry weight of the cells.

The Disruption of the Cells and the Fractionation of the Disrupted Cells—The washed cells were resuspended in distilled water in a concentration of 100 mg. wet cells per ml. of distilled water and disrupted for 10–15 minutes at 3–7° with a sonic oscillator (10 kilocycles, 100 watts).

The disrupted cells were fractionated with a refrigerated centrifuge and a Hitachi L-type ultracentrifuge as Fig. 1 shows.

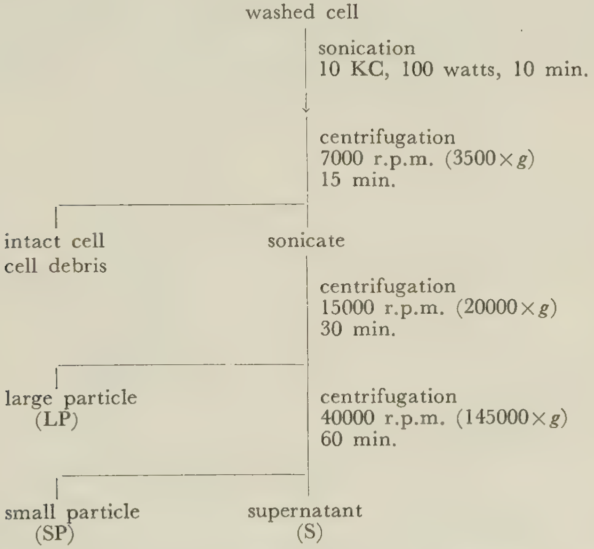

FIG. 1. The fractionation of the sonicated cells by a differential centrifugation.

Spectrophotometric Measurements—The absorption spectra of the washed or the disrupted cell suspensions, and those of the fractions obtained by the differential centrifugation of the disrupted cells were measured* by a Carry spectrophotometer making use of the opal glass method of Shibata (*15*). If necessary, the preparation was reduced by the addition of a few crystals of sodium dithionite.

The absorption spectrum of partially purified cytochrome 553 was measured ordinarily by a Beckman spectrophotometer without applying the opal glass method.

The Incorporation of Radioactive Iron—Radioactive iron was used as ferric ammonium citrate throughout the experiments. When it was used in the growth medium, the harvested cells were washed with 0.02 per cent non-radioactive ferric ammonium citrate or 0.05 *M* citrate buffer, pH 6.0, until the radioactivity of washings became negligible.

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* The measurement was performed kinkly by Dr. Shibata and Miss Kurozumi in the Tokugawa Institute for Biological Research.

The incorporation of radioactive iron into heme by the washed cells, the disrupted cells and the fractions obtained by differential centrifugation was carried out by shaking in 0.05 *M* Tris buffer, pH 7.4, at 37°. The reaction mixture shaken for given time intervals (30 and 60 minutes) was precipitated with excess of acetone. Heme was extracted with acetone-hydrochloric acid from the precipitate and purified by chloroform extraction and celite chromatography in the form of pyridine hemichrome. Radioactivity of heme was determined by a well-type scintillation counter.

The Extraction and the Purification of Cytochromes—The extraction of cytochromes was tried using citric acid (*19*) or citrate buffer (*16*) from the intact, the disrupted or the acetone-dried cells. Extracted cytochrome was purified partially by ammonium sulfate fractionation and alumina chromatography (*16*).

RESULTS

Cytochromes of Pseudomonas-P—A typical absorption spectrum of *Pseudomonas-P* cell suspension is shown in curve I of Fig. 2. As the curve shows, *Pseudomonas-P* has the absorption maxima at 421, 424, 524, 530, 553 and 560

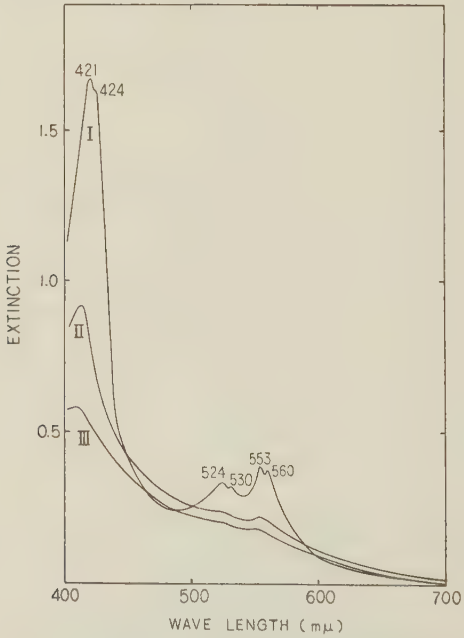

FIG. 2. Visible absorption spectra of the cells grown in different media. Each growth was carried out by shaking at 30° in 100 ml. of medium in Sakaguchi flask after 1 % inoculation. I, III and II are the spectra for the cell suspensions at a concentration of 100 mg. of wet cells per 1 ml. of the suspension, which have grown for 16 hours in bouillon-peptone, casamic acid-glucose and casamic acid-glucose with the addition of 2 mg. iron (as ferric ammonium citrate) respectively.

mμ within the range from 400 to 700 mμ. This finding seems to indicate that *Pseudomonas-P* has c-like (553 mμ as α band) and b-like (560 mμ as α band), but no a-like cytochrome, the absorption maximum of which lies at the range from 560 to 700 mμ. In other words, this microorganism has the ability to synthesize cytochromes 553 and 560, if some growth conditions are given.

The spectrum of the living cell suspension was almost the same as that of the disrupted cell suspension to which a few crystals of sodium dithionite were added. This finding seems to show that at both of cytochromes 553 and 560 are present almost in the reduced form at the stationary state of living cells. Soon after the cells were disrupted by sonication or other methods (grinding with alumina or corundum powder), the spectrum based on the reduced cytochromes disappeared.

The Extraction and the Purification of Cytochromes—Several procedures tried for the extraction of cytochromes of *Pseudomonas-P* gave only poor yield. Especially from the cells cultivated for shorter time (less than 10 hours) cytochromes were hardly extracted. Cytochrome 560 was more difficult to be extracted than 553 component. The extraction of cytochromes from the

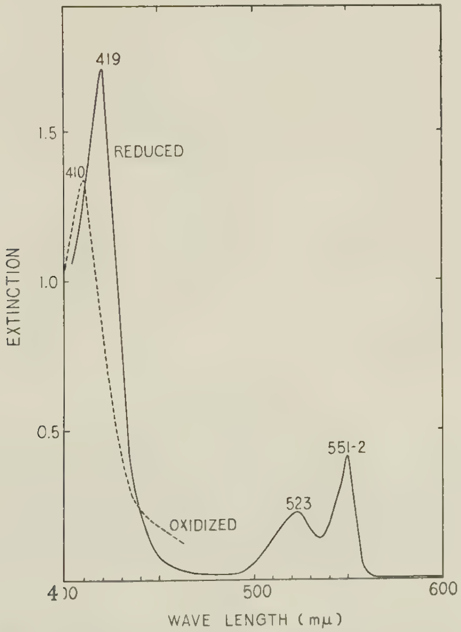

FIG. 3. The absorption spectrum of partially purified cytocorome 553*. The ratio of the extinction at 280 mμ in the oxidized form to that at 551-552 mμ in the reduced form is about 9.

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[*] Cytochrome 553 is named tentatively from the absorption maximum in the cell suspension. Purified pigment has the absorption maximum at 551-2 mμ as shown in the figure.

cells cultivated for more than 24 hours became somewhat easy and a slight but definite amount of 560 component was also extracted. Taking such findings into consideration, extraction and purification were restricted only to cytochrome 553. It started from a large amount of harvested cells obtained by a jar cultivation. Extracted cytochrome 553 was partially purified by ammonium sulfate fractionation (between 0.2 and 0.5 saturation of ammonium sulfate) and column chromatography on alumina bufferized with 0.05 *M* sodium phosphate buffer, pH 7.0. The absorption spectrum of partially purified cytochrome 553, which was measured without using the opal glass, is shown in Fig. 3. This pigment has the absorption maxima at 551-552, 523 and 419 mμ in the reduced state and shows a ratio 9 : 1 in the absorption at 280 mμ to that at 551–552 mμ. This pigment is not autoxidizable. The slight difference between the spectrum of the purified pigment and that of living cells seems to be due to the overlap of the absorptions of 553 and 560 components in living cells or the differences in the method of spectrophotometric measurements.

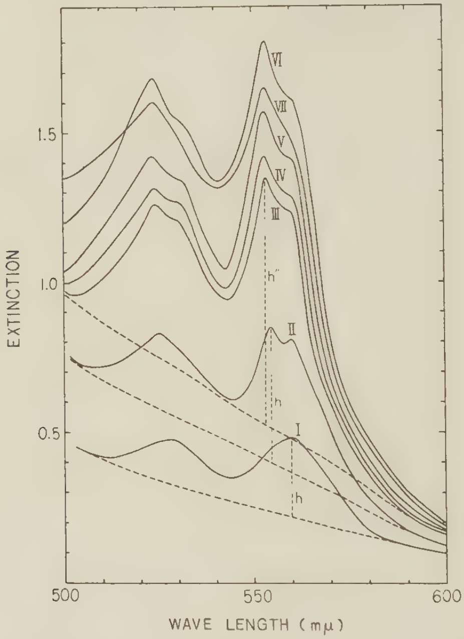

FIG. 4. Visible absorption spectra of the cells at different growth phases. The cell growth was carried out by shaking at 30° in bouillon-peptone medium in Sakaguchi flask after 1 % inoculation. Spectra correspond to cell suspensions containing 100 mg. of cells (dry weight) per ml. of suspension. I, II, III, IV, V, VI and VII are the spectra for the cells grown for 3, 4, 5, 6, 7, 10 and 14, and 24 hours, respectively.

The Variations of Cytochromes in Response to Those of the Growth Medium or the Growth Phase—The absorption spectrum of *Pseudomonas-P* cell suspension is not always similar to curve I of Fig. 2. Curve I of Fig. 2 shows the spectrum of the cells cultivated for 15 hours in bouillon-peptone medium. Curves III and II show the spectra of the cells cultivated for 15 hours in iron-poor and -rich casamic acid-glucose media, respectively. The cell growth was almost similar in three media. The result seems to indicate that cytochrome content varies in response to nutritional conditions, and that *Pseudomonas-P* is able to grow without cytochromes.

The variations of cytochromes were also observed in response to different growth phases. Fig. 4 shows the absorption spectra, measured by the opal glass method, of the washed cell suspensions at different growth phases. Fig. 5 shows the relationship between growth and cytochrome content, which is expressed tentatively by the strength of the maximum absorption at the range from 550 to 560 mμ. As the figures show, the cells incubated for 3 hours, namely the cells at the beginning of log phase (young cell), had only little cytochromes and had more 560 component than 553 component. As the growth proceeded, cytochrome content of the cell increased remarkably and the formation of cytochrome 553 surpassed that of cytochrome 560. When the cell growth reached the stationary phase (old cell), the increase of cytochrome content stopped and the spectrum of the cell suspension hardly

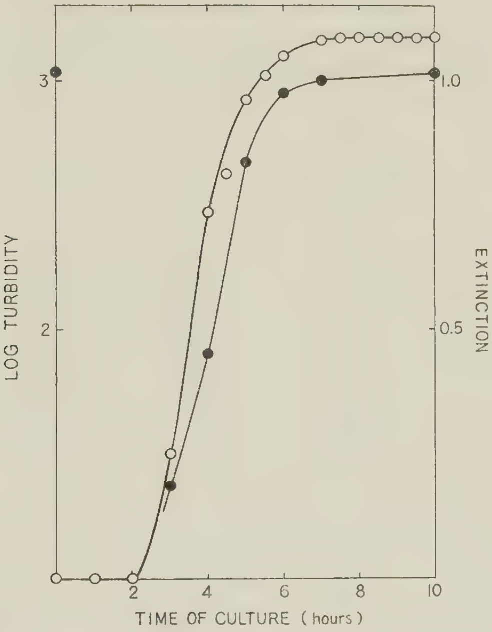

FIG. 5. The relationship between the cell growth and the cytochrome content. —○— turbidity in logarithms —●— cytochromes content corrected on the basis of the same dry weight of cells). Cytochrome content is expressed tentatively with the extinction h, h′, h″ etc. on Fig. 4 (the maximum extinction within the range from 550 to 560 mμ).

varied thereafter.

The Distribution of Cytochromes on the Cellular Components of Pseudomonas-P—The cells at different growth phases were disrupted by sonic oscillation and fractionated by differential centrifugation method shown in Fig. 1. The suspension in 0.1 *M* sodium phosphate, buffer, pH 7.0, of each fraction was measured for its absorption spectrum by the opal glass method. A few crystals of sodium dithionite was added to the suspension just before the measurement. The content of cytochromes in each fraction is shown in Table I. No qualitative difference was observed, namely the ratio of cytochrome 553 to 560 was almost similar in every fraction. However, a remarkable difference was observed in quantity of cytochromes, namely a large amount of cytochromes, both of cytochrome 553 and 560, was found in SP fraction. Moreover, the difference was most evident in the cells cultivated for 7 hours and less evident in the cells cultivated for 24 hours. This finding seems to indicate that both of cytochrome 553 and 560 are localized on a particle which belongs to SP fraction, and that such cellular structure is destroyed by the aging of cells.

TABLE I

The Distribution of Cytochromes and Nitrogen on the Fractions of Sonicated Cells

The culture was performed by shaking at 30° for different time intervals on bouillon-peptone medium in Sakaguchi flask after 1% inoculation.

time of culture	fraction	LP	SP	S
3 hours	nitrogen(1)	0.041	0.196	0.992
	cytochromes(2)	0.000	0.025	0.015
	cyt./mg N	0	0.13	0.015
7 hours	nitrogen(1)	0.145	0.604	1.450
	cytochromes(2)	0.105	0.850	0.275
	cyt./mg N	0.72	1.40	0.19
24 hours	nitrogen(1)	0.176	0.694	2.100
	cytochromes(2)	0.170	0.860	0.750
	cyt./mg N	0.97	1.24	0.36

1) Milligram nitrogen per ml. of the suspension.

2) The value is expressed as the maximum extinction of the suspension at the range from 550 to 560 mμ.

The Incorporation of Radioactive Iron into Growing Cell and the Distribution of the Radioactivity on the Cellular Components—To obtain some information on the relationship between cytochrome formation and the incorporation of iron into cells, radioactive iron was used. Radioactive iron was added into growth medium previously before the cell growth started, and the cells were harvested at appropriate time intervals and the radioactivity incorporated (into the cells) was measured. Fig. 6 shows the relationship between the cell growth and

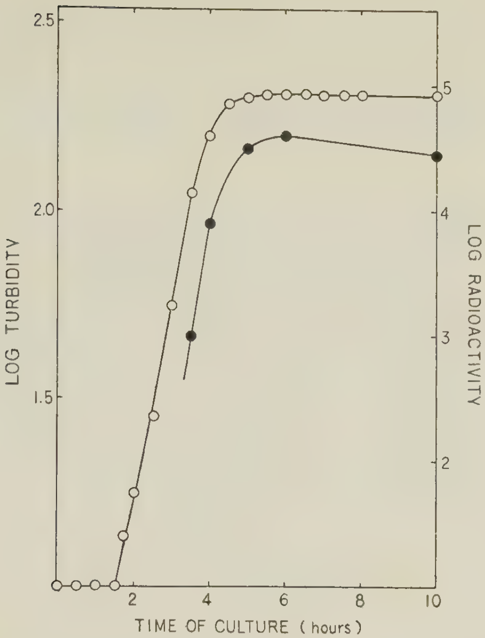

FIG. 6. The relationship between the cell growth and the uptake of radioactive iron into the cell. —○— Turbidity in logarithms, —●— radioactivity (c.p.m.) in logarithms taken up into the cells. For the culture, several Monod's test tubes were used, each of which contained 10 ml. of bouillon-peptone medium and radioactive iron (1.5×10^5 c.p.m.). The growth was carried out by shaking at 30° after 1% inoculation. Test tubes were taken out one by one at different times, and the turbidity and the radioactivity of the washed cell were measured.

TABLE II

The Uptake of Radioactive Iron into the Cells at Different Growth Phases

The culture was performed by shaking at 30° on bouillon-peptone medium in Monod's test tube after 1% inoculation. At the different times after the initiation of the culture, radioactive iron (2.0×10^5 c.p.m.) was added to each of tubes, and the culture was gone on for 30 minutes thereafter.

time of culture when radioactive iron was added to medium	incubation time with radioactive iron	change of turbidity during incubation	radioactivity taken up into cell
3.5 hours	30 minutes	135→175	1230 c.p.m.
4.5	30	205→215	2250
5.5	30	215→220	3770
21.0	30	170→170	1170

the incorporation of radioactivity into the cell. Table II shows the result of another experiment, in which radioactive iron was added into growth medium during cell growth.

The cell grown in radioactive medium was disrupted by a sonic oscillation and fractionated by differential centrifugation as mentioned above. Then, the

TABLE III

The Distribution of the Radioactivity on the Subcellular Fractions Obtained by a Differential Centrifugation

The culture was performed by shaking at 30° on bouillon-peptone medium at Sakaguchi flask after 1% inoculation and the addition of radioactive iron to medium.

expl. No.	time of culture	radioactivity added to medium	washing method	distribution of radioactivity		
				LP	SP	S
1	17.5 hours	1.5×10^{6} c.p.m.	with $FeSO_4$-KCl aq	18000 c.pm.	56000	50000
2	24	2.0×10^{6}	with $FeSO_4$-KCl aq	5300	14600	15000
3	16	4.0×10^{6}	with citrate buffer	7400	30000	30000
4	16	4.0×10^{6}	with citrate buffer	5900	25000	40000
4'*		the same as 4		980	7500	1300

* Radioactivity on the extractable heme. Extractable heme was obtained by the extraction with acetone-HCl from each of LP, SP and S fractions and purified by chloroform extraction and chromatography on celite column as pyridine heme.

TABLE IV

The Incorporation of Radioactive Iron into Heme by the Washed Cells and the Sonicated Cells

The washed and the sonicated cells were incubated with radioactive iron in 0.05 *M* Tris buffer, pH 7.4, at 37° for one hour.

expl. No.	preparation (time of culture)	washed cells			sonicated cells		
		dry weight	radioactivity incorporated into heme	radioactivity per dry weight	dry weight	radioactivity incorporated into heme	radioactivity per dry weight
1 1)	3 hours	25.8 mg.	50400c.p.m.	2000c.p.m.	37.6 mg.	18900c.p.m.	500c.p.m.
	5	28.5	59300	2080	66.8	82367	1230
	8	28.5	13300	470	23.5	33300	1410
2 1)	5				55.5	26799	480
	8				74.1	45606	620
3 2)	5	46.8	27604	590			
	8	44.0	20804	470			

1) Radioactive iron 7.7×10^{5} c.p.m. was incubated with the preparation.
2) 4.9×10^{5} c.p.m. was used.

radioactivity of each fraction was measured. In some experiment, heme was extracted from each fraction and its radioactivity was measured. The result is shown in Table III. Here, it must be noticed that the heme of cytochrome 553 is not extractable by the present extracting method, if this cytochrome component is a mammalian c-like one.

Table V

The Incorporation of Radioactive Iron into Heme by the Different Fractions of Sonicated Cell

Each of LP, SP and S fractions was incubated with radioactive iron (7×10^5 c.p.m.) in 0.05 *M* Tris buffer, pH 7.4, at 37° for 30 and 60 minutes, respectively. The cell grown for 4.5 hours, was used for the experiment.

time of incubation	radioactivity into heme		
	LP (0.090 mg. N)	SP (0.428 mg. N)	S (2.020 mg. N)
30 minutes	20000 c.p.m.	26700 c.p.m.	7000 c.p.m.
60	21800	33400	7000

The Incorporation of Radioactive Iron into Heme by the Washed Cells, the Disrupted Cells and the Cellular Components—To examine whether the incorporation of racioactive iron into heme can be observed by the washed cells, the disrupted cells and the fractions obtained by differential centrifugation, as was observed in case of growing cells, they were incubated with radioactive iron in buffer solution. Heme was extracted from the acetone-precipitate. Tables IV and V show the results of incorporation experiments, which indicate that radioactive iron can be incorporated into heme also by the cellular components, and that LP fraction is the most active per unit of nitrogen of three fractions.

DISCUSSION

Up to the present time, some observations have been presented on cytochromes of *Pseudomonas fluorescens*, which seems to have the nearest relation to *Psendomonas-P* (*18*). Fujita and Kodama (*20*) observed cytochromes 551, 562 and 598, and Smith (*21*) did cytochromes 552, 560 and 580 in *Pseudomonas fluorescens*. Recently, Lenhoff, Nicholas and Kaplan (*22*, *23*) reported the extraction of cytochrome 550 from *Pseudomonas fluorescens*. The present results indicates that *Pseudomonas-P* has cytochromes 553 and 560 and no a-like component is observed in this microorganism so far as it is examined by the present method.

With the extraction of cytochrome 550, Lenhoff and others (*22*, *23*) examined the effects of oxygen tension and of iron and molybdenum deficiency in the growth medium on the cytochrome content in *P. fluorescens.* In the present investigation, the qualitative and the quantitative changes of cytochromes in *Pseudomonas-P* in response to the cell growth were examined by a new method, that is by the opal glass method of Shibata (*15*). The following observations may be of some interest. Even under normal growth conditions, namely the conditions for this microorganism to grow normally with the formation of cytochromes, cytochromes are absent or very poor at the beginning of log phase and they are formed rapidly as the growth phase proceeds. Moreover, in their formation, cytochrome 560 component appears at first and the formation of 553 component is delayed slightly, but soon the latter gets ahead of the former. The formation rates of cytochromes are very rapid and the cells become, only within a few hours, to have cytochromes in an amount over ten times compared with the beginning of log phase. Only from the present result, the conversion from 560 component to 553 component would not be discussed. However, Ephrusse and Slonimski (*24*) suggested that cytochrome b_1 (α absorption at 560 mμ) might be a precursor of b and c pigments in baker's yeast, and Chaix, Roncoli and Petit (*25–27*) observed also the similar change in other microorganisms. Taking these observations into consideration, a possibility may be projected upon the mechanism of cytochrome formation in some microorganisms.

From the findings that the cells grown at a high oxygen tension had only traces of cytochrome pigment, whereas the cells grown at a low oxygen tension contained a large amount of the pigment, Lenhoff and others (*22*, *23*) discussed the effect of oxygen tension in determining the cytochrome content and the alternative terminal electron transfer pathway of *Pseudomonas fluorescens.* The present result may be discussed by a similar interpretation that a cell at the beginning of log phase in the growth is able to take up oxygen enough and subsequently has only traces of cytochromes, but owing to the distinguished increase of cell number as the growth phase proceeds, a cell becomes difficult to take up oxygen enough and subsequently has a large amount of cytochromes. However, in the present investigation, the cells grown in casamic acid-glucose medium, in which *Pseudomonas-P* accomplishes the same growth as in bouillon-peptone medium, has only traces of cytochromes and the addition of a large amount of ferric ions has little effect on the increase of cytochrome content. Considering the present results in addition to the investigation of Lenhoff and others (*22*, *23*), the formation of cytochromes in *Pseudomonas-P* seems to be controlled not only by oxygen and iron concentrations, but also by any other nutritional conditions.

There have been presented many discussions on the problem of cellular components in bacterial cells and their fractionation method. This problem on *Pseudomonas-P* will be discussed by Mitsui on another paper*. Concerning the distribution of cytochromes on cellular components, Tissieres, Hovenkamp and Slater (*28*) observed that, in *Azotobacter vinelandii*, cytochrome c_4 was rich in SP fraction obtained by the same differential centrifugation method as used by the present authors and that fraction was the most active in the respiration. The present result on *Pseudomonas-P*, indicates also that both of cytochromes 553 and 560 are present in SP fraction. In the case of *Pseudomonas-P*, SP fraction may be important for respiration just as in *Azotobacter vinelandii*. Moreover, it seems to be interesting that this tendency in the localization of cytochromes diminishes in the cells grown for longer period (24 hours) and at the same time the extraction of the pigment from the cell becomes easier than that from the cells grown for shorter time (7 hours). The other result, obtained from isotope experiment, may also confirm this finding.

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* Mitsui, H., and Yoshida, E., Unpublished data

It was reported by Minakami (*17*) that radioactive iron could be incorporated into heme by incubation of mammalian tissue homogenate or the cellular components. Also in *Pseudomonas-P*, the incorporation of radioactive iron into heme by the washed cells, the disrupted cells or the fractionated components was observed. Of three fractions, LP fraction is the most active per unit of nitrogen in the incorporation of radioactive iron. Considering the finding that cytochrome content is the highest in SP fraction, a possibility may be suggested that heme formed on a particle contained in LP fraction is transferred to a particle in SP fraction.

SUMMARY

1. Cytochromes of *Pseudomonas-P* were studied by the opal glass method. *Pseudomonas-P* had cytochromes 553 and 560. Cytochrome 553 was extracted and partially purified.

2. Cytochrome content in *Pseudomonas-P* varied remarkably in response to the growth phase and as well as to the growth medium.

3. The distribution of cytochromes on cellular components of *Pseudomonas-P* was examined. Both of cytochromes 553 and 560 were the most abundant in SP (small particle) fraction.

4. The incorporation of racioactive iron into growing cells of *Pseudomonas-P* and the distribution of radioactivity taken up on the cellular components were examined. The results were discussed in relation to the observations on cytochrome formation and localization.

5. The incorporation of radioactive iron into heme by the washed cells, the disrupted cells or the cellular components of *Pseudomonas-P* was examined. Of the cellular components, LP (large particle) fraction was the most active in the iron incorporation.

The authors express their thanks to Prof. S. Akabori and Prof. H. Yoshikawa for their interests during the course of this work. Thanks are also due to Dr. K. Shibata for his aid in the application of his opal glass method to this work.

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ON THE TRANSPHOSPHORYLATION REACTIONS CATALYZED BY GLUCOSE-1-PHOSPHATE PHOSPHOTRANSFERASE OF ESCHERICHIA COLI

II. ENZYMATIC PHOSPHORYLATION OF NUCLEOSIDES

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In the previous paper (*1*), the authors have reported that the purified glucose-1-phosphate (G-1-P) phosphotransferase of *E. coli* catalyzes the transphosphorylation between G-1-P and riboflavin, thereby effecting the synthesis of flavin mononucleotide. The experimental results have shown that the reaction effectively proceeds even with considerably low concentrations of the acceptor riboflavin.

The present paper describes similar investigations concerned with G-1-P phosphotransferase of *E. coli*, on the synthesis of nucleotides through transphosphorylation between G-1-P and nucleosides.

The biosynthesis of nucleotides through transphosphorylation has first been studied by Brawerman and Chargaff (*2*), using their "nucleoside phosphotransferase". However, they used crude extracts of various tissues and their results were complicated for the long reactions of incubations.

The experimental results described below, show that the G-1-P phosphotransferase of *E. coli* is capable of phosphorylation of all nucleosides tested, and in the case of adenosine the phosphorylation proceeds only in the 5′-position of the sugar moiety; they would provide some information on the biosynthesis of nucleotides in bacterial cells.

EXPERIMENTAL

Materials—Cytidine, uridine and thymidine were kindly supplied by Dr. T. Hashizume of this Department. The other compounds used in this study were commercial products.

G-1-P phosphotransferase of *E. coli* was prepared from the dried cells, as previously described (*1*).

Method—Inorganic phosphate was determined by the method of Fiske-Subbarow (*3*), and glucose by Somogyi-Nelson's method (*4*).

Nucleosides and nucleotides were separated by paper chromatography with 5 per cent Na_2HPO_4 saturated with isoamylalcohol. Detection of spots was made by ultraviolet printing technique on photographic paper (*5*), and the relative R_f values given by

Carter and Buchanan (*6*) were also used for the identification. Estimation of nucleotides were made according to Magasanik *et al.* (*7*).

Ion exchange chromatography was employed for the isolation of the products, using Dowex-1-formate column (*8*). Periodate titration was made by the procedure of Lythgoe and Todd (*9*), on a microscale.

RESULTS

Phosphorylation of Various Nucleosides by G-1-P Phosphotransferase of E. coli—The results, as shown in Table I, demonstrated that all nucleosides tested were well react with G-1-P by the catalytic action of G-1-P phosphotransferase of *E. coli*, effecting the synthesis of their nucleotides.

TABLE I

Phosphorylation of Nucleosides by G-1-P Phosphotransferase of E. coli

The reation mixture (2.5 ml.) containing 4 m*M* nucleoside, 10 m*M* G-1-P, 0.1 *M* acetate buffer (pH 5.2) and 1 ml. of the purified enzyme, was incubated at 30° for 30 minutes.

Nucleoside	Nucleotide synthesized (m*M*)	Glucose liberated (m*M*)	PR[1] (%)
Adenosine	0.126	3.77	3.35
Guanosine	0.132	3.57	3.71
Inosine	0.096	4.11	2.33
Uridine	0.094	3.95	2.38
Cytidine	0.097	3.82	2.53
Thymidine	0.081	3.98	2.08

1) Phosphorylation ratio.

It was found that purine nucleosides were phosphorylated with rather ease than pyrimidine nucleosides under the same conditions of the reaction.

The phosphorylation ratio, that is the molar ratio of the phosphate transferred to the glucose liberated, was chiefly a function of the concentration of acceptor. However, the nucleosides produced a slight inhibition of the rate of G-1-P splitting, as shown in Table II.

Identification of the Reaction Products—In a large scale experiment, 100 mg. of adenosine was incubated with 300 mg. of G-1-P and 10 mg. of the enzyme in 40 ml. of 5×10^{-2} *M* acetate buffer (pH 5.2), at 37° for 2 hours.

The reaction was stopped by heating for 5 minutes in a boiling water bath, the precipitate was removed by centrifuging, then the supernatant was passed through Dowex-1-formate column.

The product, adenosine monophosphate, was eluted with 0.15 *M* formic

TABLE II

Effect of Acceptor Concentrations

The reaction conditions were similar as those of Table and various concentrations of adenosine were used.

Concentration of Adenosine (m*M*)	Adenosine monophosphate synthesized (m*M*)	Glucose liberated (m*M*)	PR (%)
0	—	3.78	—
2	0.064	3.78	1.69
4	0.126	3.76	3.35
8	0.231	3.69	6.28
16	0.382	3.55	10.79

acid, and again absorbed on a charcoal column (*10*). The column was washed with water, and adenosine monophosphate was eluted with 50 per cent ethylalcohol containing 3 per cent NH_4OH. The eluate was concentrated to dryness *in vacuo*. By the procedures described above, approximately 3.4 g. of adenosine monophosphate was isolated.

The isolated adenosine monophosphate, which was chromatographically homogeneous, was submitted to chemical analysis, and found to have an equimolar ratio of organic phosphorus to nucleoside. The molar extinction coefficient (ε) at 260 mμ was about 15.2; a value of 15.0 was obtained for the commercial product* of adenosine-5′-phosphate.

The position of the absorption maximum and the ratio of optical densities, 280 mμ/260 mμ, were identical for both products.

Molar ratio of periodate uptake was found to be 0.93 to 1.20, indicating that 5′-isomer of adenosine monophosphate was synthesized.

DISCUSSION

Since riboflavin and its analogues were found to react with G-1-P in the presence of G-1-P phosphotransferase of *E. coli*, it was of interest to investigate whether natural nucleosides could be similarly converted to the corresponding nucleotides by the action of the enzyme. The experimental results described in this paper, show that G-1-P phosphotransferase effectively catalyzes the synthesis of mononucleotides through transphosphorylation between G-1-P and nucleosides.

It is shown that the phosphorylation ratio is chiefly a function of the acceptor concentration, and of the affinity of the enzyme for the acceptor, especially for its configuration or for groups adjacent to hydroxyl.

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* The product of Zellstofffabrik Waldhof, Germany.

The specificity of the enzyme to the product synthesized is noteworthy. As indicated from the result obtained in the case of adenosine monophosphate, 5′-isomers of nucleotides would be the products formed by G-1-P phosphotransferase, and the phosphorylation would proceed preferentially in the 5′-position of sugar moeity. This suggests that the acceptor nucleoside is absorbed at the specific site of the enzyme surface (*11*). Brawerman and Chargaff (*2*) found, on the other hand, that about equal amounts of 5′- and 3′-cytidylic acid were formed with cytidine, by their "nucleoside phosphotransferase", indicating that their enzyme was different from G-1-P phosphotransferase of the present authors, in the position of phosphorylation.

G-1-P phosphotransferase activity is found to be enriched in the bacterial cells which grow in a medium containing maltose, and the most increase in activity is observed in the stage of rapid growth. This indicates that G-1-P phosphotransferase is associated with growth processes and that the dividing cells utilize G-1-P in nucleotide synthesis.

SUMMARY

1. It was shown that G-1-P phosphotransferase of *E. coli* catalyzed the transphosphorylation from G-1-P to nucleosides, synthesizing the corresponding nucleotides.

2. The experimental results using adenosine, indicated that the enzyme specifically phosphorylated the 5′-position of sugar moiety of nucleoside.

3. A possible role of G-1-P phosphotransferase in the biosynthesis of nucleotides was discussed.

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STUDIES ON BACTERIAL AMYLASE

III. ON THE AMINO-TERMINAL PEPTIDE OF BACTERIAL AMYLASE

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(Received for publication, August 17, 1959)

In the previous paper (*1*), it was shown that the N-terminal amino acid of the amylase produced by *Bacillus subtilis N* (BA) was valine. Comparing this result with that of Taka-amylase A (*2*), it was speculated that the N-terminal amino acid of α-amylase was not directly connected with the enzymic activity. A comparison of the N-terminal sequence of BA with that of Taka-amylase A (*3*, *4*) is thus made and it represents the subject of the present contribution.

Dinitrophenyl (DNP) protein, which was prepared by the Sanger's technique (*5*), was partially hydrolyzed with concentrated hydrochloric acid or with the protease of *Streptomyces griseus* (*Streptomyces protease G*). The DNP-peptides produced were fractionated and purified by Amberlite XE-64 (hydrogen form) column, silica gel column and paper chromatography, and amino acid sequence of each purified DNP-peptide was elucidated. For the N-terminal pentapeptide, the following sequence was deduced: (H)Val·Asp·Gly·Glu·Ser·

EXPERIMENTAL

Materials—The lyophilized BA was kindly supplied by Nagase Ltd. Co. Anhydrous hydrazine was prepared according to Stähler's method (*6*). The analysis was 98-100 per cent of N_2H_4.

Streptomyces protease G (*7*) was generously supplied by Dr. M. Nomoto of the Institute of physical and chemical research and trypsin was obtained Worthington Biochemical Corporation, Freehold, N. J.. They were used without further purification.

DNP-BA was prepared in a similar method as described previously (*1*).

Partial Hydrolysis of DNP-BA with Concentrated Hydrochloric Acid—About 400-1,000 mg. of DNP-BA was hydrolyzed with 20-50 ml. of concentrated hydrochloric acid at 37° for 7 or 8 days. The reaction mixture was diluted about sixfold with water and DNP-peptides were extracted with ether and ethylacetate. The solvents were evaporated *in vacuo*.

Enzymic Digestion—Either of trypsin or *Streptomyces protease G* was added to the suspension of DNP-BA in hundredfold of $M/10$ veronal buffer of pH 8.0 with the enzyme to substrate ratio 1 : 100. The mixture was incubated at 37° for 24 hours. In the case of bacterial protease digestion, an additional enzyme was added to the mixture to make the final enzyme concentration 2 per cent and incubation was continued for further 48 hours. The digestion was stopped with the addition of 6 *N* HCl to pH 1 and the insoluble material was centrifuged off. The supernatant was shaken successively with ether and ethylacetate and the residue obtained after removal of the solvent was used for subsequent fractionation. In the tryptic digestion, any DNP-peptide did not go into ether phase.

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During digestion, the extent of the hydrolysis was followed with the ninhydrin reaction.

Fractionation of N-Terminal DNP-Peptides

Paper Chromatography—The ether-soluble fraction was fractionated by the aid of paper chromatography with 0.1 per cent NH_3 saturated butanol (NH_3-butanol). An instance of the paper chromatogram is shown in Fig. 1. Each band was extracted with 1 per cent

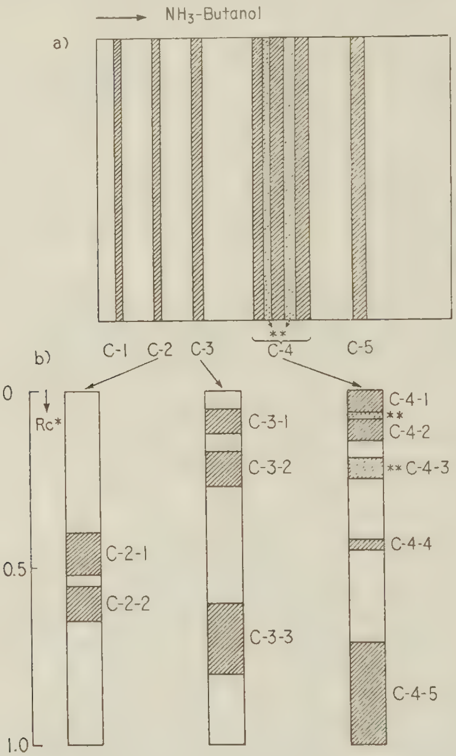

FIG. 1. a) Paper chromatogram of DNP-N-terminal fraction extracted with ether from acid-hydrolysate of DNP-BA.

b) Rechromatograms of bands, C-2, C-3 and C-4, in a) by silica gel columns with 10 BC-10 A.

$$* \ \mathrm{Rc} = \frac{\text{The distance from the top of silica gel to the band}}{\text{The distance from the top of silica gel to the front}}$$

** Dotted line means a faint band.

$NaHCO_3$ at 55–60° for 15 minutes; the solution was acidified with 2 N HCl (thymol blue-red) and DNP-peptide was extracted with ethylacetate. The organic solvent was evaporated *in vacuo* and the residue was rechromatographed on paper with NH_3-butanol or on silica gel column with butanol-chloroform-acetic acid-water (1 : 9 : 1 : 9 vol./vol.; abbreviation: 10 BC-10 A). Each DNP-derivative was extracted with ethylacetate and methanol from the cut silica gel.

Amberlite XE-64 Column Chromatography—The chromatography of DNP-peptides on the resin column was performed in a similar manner as reported by Seki (*8*). The dried ether-soluble fraction was dissolved in 1 per cent HCl-methylethylketone (78 : 22, vol.) and applied to XE-64 column (hydrogen form, 2.0×38 cm.) at a flow rate about 15 ml. per hour.

Five or ten ml. aliquots of the effluent were collected automatically, three successive fractions were combined, and the amount of DNP-peptide in each combined fraction was estimated at 400 mμ by A. K. A. photoelectric colorimeter. The elution curves of the hydrolysates with HCl and with *Streptomyces protease G* are shown in Fig. 2 and Fig. 3, respectively. Further purification of each fraction was carried out by paper chromatography with NH_3-butanol and silica gel column chromatography with 10 BC-10 A.

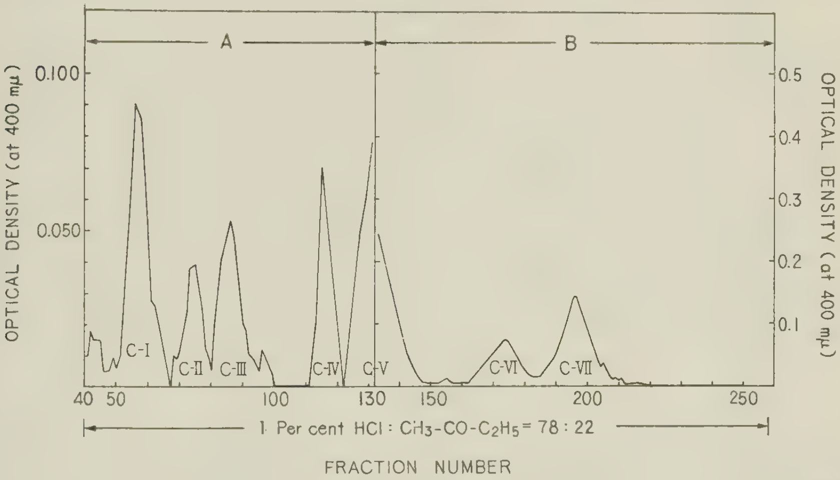

FIG. 2. Elution curve of the hydrolysate with hydrochloric acid from a column of IRC-50, 2.0×38 cm. (the hydrogen form). The effluent was collected in about 5 ml. fractions.

(A) The measurement was carried out by A. K. A. photoelectric colorimeter.

(B) The measurement was carried out by Shimazu photoelectric spectrophotometer.

Amino Acid Sequence of the N-Terminal DNP-Peptides—N-Terminal amino acid analysis was carried out by the 1-fluoro-2, 4-dinitrobenzene (FDNB) method.

Each purified DNP-peptide was hydrolyzed with an adequate amount of constant boiling hydrochloric acid in a sealed tube at 105-110° for 17-24 hours. The hydrolysate was diluted threefold with water, DNP-amino acid was extracted with ether and the solvent was evaporated *in vacuo*. The N-terminal DNP-amino acid was analyzed by two-dimensional paper chromatography, as described by Koch *et al.* (*9*).

The hydrolysate of DNP-peptide, which contained N-terminal valine, was shaken with ether, and the aqueous phase was used for amino acid analysis of the DNP-peptide by paper chromatography as suggested by Wolfe (*10*). The residue was treated with FDNB as usual, DNP-amino acids produced were separated by two-dimensional paper chromatography as described above and determined colorimetrically (at 360 mμ, Shimazu photoelectric spectrophotometer). The identification of DNP-acidic amino acids was performed by silica gel column chromatography with 10 BC-10 A.

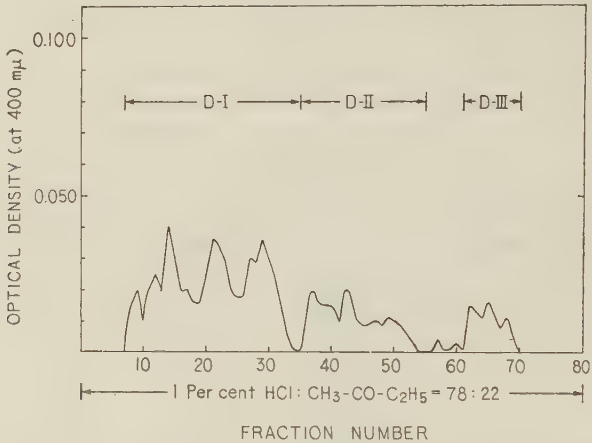

FIG. 3. Elution curve of the hydrolysate with *Streptomyces protease G* from a column of IRC-50, 2.0×38 cm. (the hydrogen form). The effluent was collected in about 10 ml. fractions.

The C-terminal amino acid was characterized and estimated by the hydrazinolysis method (*11*), the identification of DNP-amino acids being performed by paper chromatography.

RESULTS

Three acid and one enzyme hydrolysates of DNP-BA were prepared and ether-soluble N-terminal DNP-peptides in the hydrolysates were fractionated by either of paper chromatography, Amberlite XE-64 or silica gel column chromatography. Some of DNP-peptides were not homogeneous on a single fractionation and they were refractionated by the aid of another method. Fractionation system of the DNP-peptides and fractionated components are tabulated (Table I). As is seen in Table I some of peptides were still heterogeneous and they were not analyzed in detail.

N-Terminal group of all of the purified DNP-peptides was analysed but C-terminal analysis was made only on a few peptides. Amino acid compositions and probable structures of the peptides analysed are illustrated in Table II. Whether acidic amino acid residue in N-terminal peptide is amide or not has not been examined. Summarizing Table II, dipeptide, Val·Asp (C-2-1), tripeptide, Val·Asp·Gly or Val(Asp, Gly) (A-3, B-3, B-III, C-3-1, C-4-2, C-4-4, C-V and D-II-1), tetrapeptide, Val(Asp, Gly, Glu) (A-2), pentapeptide, Val(Asp, Gly, Glu, Ser) (A-1) and much longer peptides were identified. The origin of the DNP-glutamylpeptides contaminating some of the N-terminal

Table I

Fractionation of N-Terminal DNP-Peptides of the Bacterial Amylase Produced by Partial Hydrolysis

Hydrolysis conditions	Chromatographic fractionation method			Remarks
	XE-64 column	Paper	Silica gel colum	
conc. HCl 37° 7 days		A-1		
		A-2		
		A-3		
		A-4		DNA-NH$_2$[1]
conc. HCl 37° 8 days	B-I			slight[2]
	B-II			slight
	B-III			
	B-IV			not homogeneous
		B-1		=A-1[3]
		B-2		=A-2
		B-3		=A-3
		B-4		DNP-OH[4]
		B-5		DNA-NH$_2$
conc. HCl 38° 7 days		C-1		contaminated[5]
		C-2 →	C-2-1	
			C-2-2	
		C-3 →	C-3-1	
			C-3-2	contaminated
			C-3-3	contaminated
		C-4 →	C-4-1	mixture[6]
			C-4-2	
			C-4-3	slight
			C-4-4	
			C-4-5	contaminated
		C-5		DNA-NH$_2$
	C-I			DNP-OH
	C-II			contaminated
	C-III			
	C-IV			contaminated
	C-V			
	C-VI			contaminated
	C-VII			DNA-NH$_2$
Protease 37° 72 hrs.	D-I →	D-I-1		
		D-I-2		DNP-OH
		D-I-3		DNA-NH$_2$
	D-II →	D-II-1		
		D-II-2		
	D-III			

1) Dinitroaniline.

2) Amount of these bands was slight and it was not analysed.

3) Judging from the position on the paper chromatogram, B-1, B-2 and B-3 appeared to be the same with A-1, A-2 and A-3, respectively. Consequently, B-1 and B-2 were not analysed.

4) Dinitrophenol.

5) In addition to DNP-valine a small amount of DNP-glutamic acid was found after hydrolysis of DNP-peptides and further analyses of these peptides have not been explored.

6) Band C-4-1 stayed on the top of the column and it was not analysed.

note: A number of each band on all chromatograms was given in the order of increasing *Rc* and R_f.

TABLE II

Analysis of N-Terminal DNP-Peptides

<table>
<tr><th rowspan="2">Sample</th><th rowspan="2">N-Terminal</th><th colspan="10">Amino acid composition of acid-soluble fraction (molar ratio)</th><th rowspan="2">C-Terminal</th><th rowspan="2">Probable structure</th></tr>
<tr><th>Asp</th><th>Glu</th><th>Gly</th><th>Ser</th><th>Ala</th><th>Val</th><th>Leu or Ileu</th><th>Thr</th><th>Phe</th><th>Lys</th></tr>
<tr><td>C-2-1</td><td>Val</td><td>1.0</td><td>t[1]</td><td>t</td><td>t</td><td>t</td><td>t</td><td>t</td><td></td><td></td><td>t</td><td></td><td>Val·Asp</td></tr>
<tr><td>A-3
D-II-1</td><td>Val</td><td>1.2</td><td></td><td>1.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>Val·(Asp, Gly)</td></tr>
<tr><td>B-3
C-4-4</td><td>Val</td><td>0.8</td><td></td><td>1.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>Gly</td><td>Val·Asp·Gly</td></tr>
<tr><td>B-III</td><td>Val(1.1)[2]</td><td>1.0</td><td></td><td>1.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>Gly</td><td>Val·Asp·Gly</td></tr>
<tr><td>C-3-1</td><td>Val</td><td>1.1</td><td></td><td>1.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>Val·(Asp, Gly)</td></tr>
<tr><td>C-4-2</td><td>Val</td><td colspan="2">1.2</td><td>1.0</td><td>$\underline{t}$[3]</td><td>$\underline{t}$</td><td>$\underline{t}$</td><td></td><td></td><td></td><td></td><td></td><td>Val·(Asp or Glu, Gly)</td></tr>
<tr><td>C-V</td><td>Val</td><td>1.0</td><td>t</td><td>1.0</td><td>$\underline{t}$</td><td>t</td><td>t</td><td>$\underline{t}$</td><td></td><td>$\underline{t}$</td><td></td><td></td><td>Val·(Asp, Gly)</td></tr>
<tr><td>A-2</td><td>Val</td><td colspan="2">1.8</td><td>1.0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>Val·(Asp, Gly, Glu)</td></tr>
<tr><td>A-1</td><td>Val</td><td colspan="2">2.0</td><td>1.0</td><td>1.2</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>Val·(Asp, Gly, Glu, Ser)</td></tr>
<tr><td>D-I-1</td><td>Val</td><td colspan="2">1.8</td><td>1.0</td><td>0.6</td><td>t</td><td>0.6</td><td></td><td></td><td></td><td></td><td></td><td>Val·(Asp, Gly, Glu, Ser, Val)</td></tr>
<tr><td>D-II-2</td><td>Val</td><td colspan="2">2.4</td><td>1.0</td><td>0.9</td><td>t</td><td colspan="2">1.8</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>C-III</td><td>Val</td><td colspan="2">3.6</td><td>1.0</td><td>1.2</td><td>1.2</td><td>1.5</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>C-2-2</td><td>Val</td><td colspan="2">3.1</td><td>1.0</td><td>$\underline{0.9}$</td><td>$\underline{0.6}$</td><td colspan="2">1.6</td><td>$\underline{1.0}$</td><td>1.1</td><td></td><td></td><td></td></tr>
<tr><td>D-III</td><td>Val</td><td colspan="2">2.0</td><td>1.0</td><td>0.9</td><td>1.0</td><td>1.0</td><td>$\underline{1.0}$</td><td></td><td>$\underline{1.1}$</td><td>$\underline{1.0}$</td><td></td><td>Val·(Asp, Gly, Glu, Ser, Ala, Val)</td></tr>
</table>

1) "t" means a trace amount of amino acid.
2) This value shows molar ratio to glycine.
3) The "underlines" mean that these amino acids can be recognized only on paper chromatograms of DNP-amino acids.

DNP-valylpeptides in Table I (C-3-2, C-3-3, C-4-5, C-5, C-II, C-IV and C-VI) is unknown. From these results the following amino acid sequence in the N-terminal group of BA is derived.

Val-Asp·[or Asp(NH_2)]-Gly-Glu·[or Glu(NH_2)]-Ser···

DISCUSSION

Judging from the Rc value of C-3-1 which corresponded to that of C-4-2, both may be the same tripeptide and main component of C-4-2 is tripeptide, (Val, Asp, Gly). The difference of the Rc-values between C-4-2 (=C-3-1) and C-4-4 may be due to that aspartic acid residue in one of these peptides is amide.

It was hard to prove the amino acid sequence beyond serine in neighbour of N-terminus of BA. But the data for peptides, D-I-1, D-II-2, C-III, C-2-2 and D-III, suggested that the subsequent amino acids were Ala, Val and Leu or Ileu.

In addition, one purified DNP-peptide (D-III) obtained from the protease digestion of DNP-BA was treated with Ehrlich's reagent (*12*), and it was demonstrated that this N-terminal DNP-peptide extracted with ether (at least a hepta-peptide) didn't contain tryptophan.

Although these peptides contained trace amounts of contaminated peptide, and if constituent amino acids in these peptides are considered, it is assumed that the N-terminal peptide of BA is Val-Asp·[or Asp(NH_2)]-Gly-Glu·[or Glu(NH_2)]-Ser-(Ala, Val, Leu or Ileu)····.

The fact that no N-terminal DNP-peptides could be extracted with ether from tryptic digest might suggest that arginine seemed not to be present in the neighbour of N-terminus.

Yoshida and Tobita (*13*) has shown that the amino acid sequence of the N-terminal peptide of BA is Val-Asp-Gly-Glu-(Ser, Ala, Val)-(Leu, Ileu, Thr, Gly)-Lys····. Our data has been confirmed by this result.

It is interesting that the amino acid composition of the N-terminal peptides of both BA and Taka-amylase A is the same at least to the fourth amino acid except for N-terminus.

As mentioned above, Narita *et al.* (*3*) and Tsugita (*4*) reported that the N-terminal peptide of Taka-amylase A was Ala-Gly-Asp·[or Asp (NH_2)]-Glu·[or Glu(NH_2)]-Ser-Ala-Leu-(Thr)·····. In comparison with these results, it has been shown that BA has a similar amino acid sequence to Taka-amylase A in the N-terminal region, except for the following two point; 1) N-terminal amino acid of BA(Val) is different from that of Taka-amylase A (Ala). 2) The amino acid sequence (-Asp-Gly-) next to N-terminus in BA is the reverse of that (-Gly-Asp-) of Taka-amylase A.

Narita *et al.* showed that no N-terminal DNP-peptide could be obtained except DNP-alanine by a mild hydrolysis of DNP-Taka-amylase A with hydrochloric acid. On the other hand, the authors were able to obtain N-terminal peptides of DNP-BA by hydrolysis with concentrated hydrochloric acid. The fact could be explained by the difference of N-terminal amino acids between two α-amylases. In addition, the present results show that the N-terminal DNP-peptides can be obtained by the hydrolysis with *Streptomyces protease G* and that Seki's method (*8*) may be applicable to the fractionation of small DNP-peptides. The results obtained in the present investigation might suggest that the N-terminal peptide of α-amylase has a certain relation to the α-amylase activity, but the evidence is not yet sufficient to derive any decisive conclusion.

SUMMARY

The N-terminal peptides of DNP-bacterial amylase were obtained by partial hydrolysis with concentrated hydrochloric acid and *Streptomyces protease G*. The amino acid sequence of the N-terminal DNP-peptide is proposed to be Val-Asp·[or Asp(NH_2)]-Gly-Glu·[or Glu(NH_2)]-Ser-(Ala,Val,Leu or Ileu)····. This result was discussed in comparison with that of Taka-amylase A.

We would like to express gratitude to Prof. S. Akabori for his kind guidance throughout this investigation and also to Nagase Ltd. Co. for the gift of bacterial amylase and to Dr. M. Nomoto for the supply of *Streptomyces protease G*.

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STUDIES ON FREE AMINO ACIDS IN HUMAN BRAIN

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The significant differences of the amounts of free amino acids in the brains of various species of vertebrate (*1*) and of their distribution in various parts of dog brain (*2*) have recently been reported from this laboratory. Berl *et al.* (*3*) have reported also the concentration of several amino acids in various parts of rat brain. The change of each amino acid content of mouse brain at various developmental stages has been observed by Roberts *et al.* (*4*). Tallan *et al.* (*5*) have recently found the presence of a large amount of cystathionine in human brain.

In the present study, analysis of free amino acids in the human brains has been undertaken in the cases of both the normal and pathological, and in various parts of the mature and fetal brains.

EXPERIMENTALS

Preparation of Tissue Extracts—The protein-free extract from about 1 g. brain tissue was prepared with 90 per cent ethanol as described previously (*1*).

Autopsy Samples—Sample tissues were obtained from 19-year old healthy and stout young man killed by a stab in his abdomen by the dagger. The preparation of tissue extract was made 12 hours after death. Also the brains of artificially aborted fetuses on the fifth gravid month and on the eighth gravid month were used. Tissue treatments were carried out at 30 minutes and 3 hours after death, respectively.

Biopsy Samples—Brain tissues were from 3-year old girl and from 17-year old boy both with brain tumor; and from 18-year old girl with tuberous sclerosis; and from 35-year old female with genuine epilepsy. Some pieces of the normal cerebral cortex surrounding the tumor were sectioned during the surgical operation and immediately frozen in dry-ice acetone.

Chromatographic Analysis—The extracts were analyzed on 150×0.9 cm. column of Dowex 50-X4 (200-400 mesh) by the method of Moore and Stein (*6*) as described previously (*1*).

RESULTS

The distribution patterns of amino acids in various parts of the brain of 19-year old boy died of an accident is presented in Table I. The values of each amino acid and the distribution pattern resemble to those of the dog brain previously described (*2*). Nevertheless, the observed values of threonine, serine, and the sum of glycine and alanine, and leucine in this instance are higher than those in the dog brain throughout the parts examined. It may be a postmortem artifact, because the sample was obtained at autopsy 12 hours after death and the proteolysis in tissue might have proceeded. The

TABLE I

Concentration of Free Amino Acid in Various Areas of the Human Brain

The brain was from a 19-year healthy and stout boy died of an accident. The preparation of tissue extract was carried out at 12 hours after death.

Values expressed in mg. per 100 g. wet weight.

Constituent	Frontal Lobe	Corpus Callosum	Caudate Nucleus	Globus Pallidus	Thalamus	Hypothalamus	Medulla
Glycerophosphoethanolamine	22.5	23.4	24.9	16.6	23.6	24.8	50.7
Phosphoethanolamine	16.5	—	14.6	4.6	10.8	5.4	2.8
Taurine	18.1	11.9	15.1	14.1	9.2	14.9	13.5
Aspartic Acid	38.2	10.7	26.8	33.7	38.8	39.2	16.2
Threonine	6.3	6.4	9.1	18.0	12.4	9.9	10.0
Serine	12.7	11.0	23.2	18.9	18.3	15.7	22.9
Glutamic Acid	154.2	61.5	180.5	146.2	135.8	103.5	94.0
Glycine & Alanine	34.3	22.6	39.2	49.8	42.7	36.4	54.4
Cystathionine	1.7	9.5	2.3	—	—	3.3	5.3
Isoleucine	2.4	3.6	4.0	—	—	1.8	7.8
Leucine	7.3	5.2	11.4	9.4	9.3	11.6	15.6
Gaba	21.0	7.2	27.4	70.5	39.5	48.0	22.0

—: Not detected.

TABLE II

Concentration of Free Amino Acid in Various Areas of the Human Fetal Brain

The brain of a fetus on the fifth gravid month was obtained from the case of artificial abortion. The preparation of tissue extract was carried out at 30 minutes after death.

Values expressed in mg. per 100 g. wet weight.

Constituent	Frontal Lobe	Occipital Lobe	Caudate Nucleus	Hypothalamus	Pons & Medulla	Cerebellum
Glycerophosphoethanolamine	12.6	21.7	12.0	34.9	27.5	15.1
Phosphoethanolamine	104.8	74.0	131.0	94.2	58.8	56.2
Taurine	57.3	60.0	62.8	51.5	47.4	45.1
Aspartic Acid	10.3	6.8	8.6	10.1	7.8	7.5
Threonine	18.1	8.1	11.0	10.0	8.4	8.2
Serine	11.8	11.5	14.8	16.3	10.9	9.3
Glutamic Acid	46.4	52.3	57.2	53.6	52.1	40.4
Glycine	8.5	8.5	10.2	14.2	12.3	7.4
Alanine	12.9	12.9	15.7	15.8	11.1	11.1
Isoleucine	1.6	2.9	1.8	3.6	1.3	3.0
Leucine	2.8		3.4	2.8	2.8	3.7
Tyrosine	5.3	2.7	4.7	4.9	6.7	3.7
Gaba	2.7	4.5	15.5	34.0	16.9	12.5
N-Acetylaspartic Acid	19.6	38.1	27.8	47.4	28.4	20.1

values of cystathionine in this sample are remarkably low throughout the parts examined. The finding that the value of cystathionine in the corpus callosum is higher than that in the frontal lobe resembles to the results obtained with the dog brain (*2*). The largest amount of γ-aminobutyric acid (Gaba) was found in the globus pallidus, followed by that in the hypothalamus.

The distribution patterns of amino acids in various areas of the fetal brain on the fifth and eighth gravid months are presented in Tables II and III, respectively. Relatively smaller amounts of aspartic acid, glutamic acid, Gaba and N-acetylaspartic acid, and larger amounts of phosphoethanolamine, taurine, threonine, glycine, alanine and tyrosine were found throughout the various parts of fetal brains. In these brains, with some variations according to sites, glutamic acid, Gaba and N-acetylaspartic acid generally tend to increase during the growth of the brain, while phosphoethanolamine, taurine and tyrosine tend to decrease. These coincide with the findings reported by Roberts *et al.* (*4*) of the mouse brain. The cystathionine was not found in the brain on the fifth gravid month but detected only in the combined sample of the pons and medulla of the brain on the eighth gravid month. The largest amount of Gaba was found in the hypothalamus throughout tested parts of the both brains.

Table III

Concentration of Free Amino Acid in Various Areas of the Human Fetal Brain

The brain of a fetus on the eighth gravid month was obtained from the case of artificial abortion. The preparation of tissue extract was carried out at 3 hours after death.

Values expressed in mg. per 100 g. wet weight.

Constituent	Frontal Lobe	Temporal Lobe	Caudate Nucleus	Thalamus	Hypothalamus	Pons & Medulla	Cerebellum
Glycerophosphoethanolamine	27.4	21.7	21.8	—1)	17.9	20.2	8.3
Phosphoethanolamine	93.5	97.4	119.2	—1)	54.0	89.5	72.8
Taurine	71.3	22.5	35.9	49.5	36.4	23.8	32.7
Aspartic Acid	8.5	6.8	8.3	11.8	8.0	15.7	8.1
Threonine	13.3	11.0	16.2	12.5	9.7	10.8	12.4
Serine	10.5	9.9	14.8	19.4	11.8	11.2	11.1
Glutamic Acid	66.0	47.5	45.5	77.2	59.2	68.2	42.0
Glycine	11.5	4.1	5.5	6.1	5.7	5.7	4.8
Alanine	11.0	16.9	9.4	23.5	16.0	13.8	8.4
Cystathionine	—	—	—	—	—	14.1	—
Isoleucine	2.3	2.4	1.5	1.4	2.9	4.6	2.7
Leucine	2.2	2.2	2.8			2.8	2.0
Tyrosine	3.7	6.6	—	—1)	2.5	3.2	1.6
Gaba	6.3	10.8	9.7	17.9	33.2	12.9	18.7
N-Acetylaspartic Acid	33.8	25.8	19.6	—1)	36.7	35.0	14.8

—: Not detected. —1): Not examined.

The results of biopsy samples are presented in Table IV. The most marked finding is the presence of cystathionine in a very large amount in the brains of patients suffering from brain tumors, including the tuberous sclerosis. The value in the genuine epilepsy is much smaller than the others. The smaller amounts of aspartic acid in the cases 2 and 4, of glutamic acid in the case 2 and of Gaba in the whole are obtained. Except these, the values of each amino acid obtained in the fresh tissues of the human brain are much the same as those of the dog brain and differ considerably from those of rat (*1*).

TABLE IV

Concentration of Free Amino Acid in Human Brains

Fresh cerebral cortexes were obtained at the time of the surgical operation in three patients with cerebral tumors and one with genuine epilepsy. In the case of brain tumors, the normal cerebral cortexes surrounding the tumors were the test materials. (*1*) 3-year old girl with the brain tumor, (*2*) 17-year old boy with the brain tumor, (*3*) 18-year old girl with the tuberous sclerosis, (*4*) 35-year old woman with the genuine epilepsy.

Vlaues expressed in mg. per 100 g. wet weight.

Constituent	(1)	(2)	(3)	(4)
Glycerophosphoethanolamine	11.1	10.2	16.0	39.3
Phosphoethanolamine	29.6	10.3	23.8	5.3
Taurine	16.7	9.0	10.3	14.3
Aspartic Acid	24.6	18.0	23.8	9.8
Threonine	3.6	2.4	1.4	9.8
Serine	10.6	5.4	6.1	2.3
Glutamic Acid	139.3	92.6	126.8	118.0
Glycine	4.6	2.6	4.6	7.0
Alanine	5.6	2.9	5.3	1.7
Cystathionine	43.2	19.4	46.0	3.5
Isoleucine	2.2	—	—	1.9
Leucine		—	—	2.5
Gaba	7.4	7.5	11.1	11.4
N-Acetylaspartic Acid	106.0	—1)	101.9	67.2

— : Not detected. —1) : Not examined.

DISCUSSION

Tallan *et al.* (*5*) have found the large amounts of cystathionine in human brains from autopsies died of various diseases. They discussed the possibility of the postmortem artifact, but we found cystathionine in high concentration in the fresh tissue taken during the surgical operation. It was the case of tissues surrounding tumors, though they were macroscopically not affected by infiltration, the tested materials cannot be considered quite intact tissues. In the tissue of genuine epilepsy and accidental death, the values were very low. From these facts it may be assumed that cystathionine would accumulate under certain pathological conditions, *e.g.*, dying of illness, or suffering from brain tumors. Hope (*7*) has reported the accumulation of cystathionine in the rat brain of pyridoxine deficiency. Cystathionase has been reported by Binkley *et al.* (*8*) to be a pyridoxal-dependent enzyme, and under certain pathological conditions, the cystathionine metabolism may be blocked at this stage. This assumption, however, does not explain its presence in a relatively large amount in the monkey brain, *e.g.*, 12.8 mg. per cent found by Tallan *et al.* (*5*) and 10.1 mg. per cent of our unpublished result. It may be assumed that our values in the accidental death and genuine epilepsy are lower than the normal value, and the normal value of the human brain is identical with that of the monkey. The results of fetal brains indicate that cystathionine tends to increase during the growth of brain.

The large amount of Gaba was found in the globus pallidus and hypothalamus. Lowe *et al.* (*9*) have indicated that these regions have a high activity of glutamic decarboxylase. Albers *et al.* (*10*) found the highest activity in the hypothalamus and mesencephalon. They reported also the distribution of glutamic-γ-aminobutyric transaminase in the brain (*11*). The ratios of transaminase to decarboxylase activity varied markedly according to the various parts of the brain. They discussed the possibility that the local steady-state concentrations of Gaba might be correlated with these ratios. The ratio was the lowest in the substantia nigra and relatively low in the globus pallidus.

The occurrence of Gaba in relatively large amount in the globus pallidus and hypothalamus may be of some interest, considering the fact that this amino acid has profound inhibitory effects on the transmission of impulse in the central nervous system (Florey (*12*), Bazemore *et al.* (*13*), Killam (*14*)).

SUMMARY

1. The analysis of amino acids in the protein-free extracts of the human brain has been carried out by means of ion exchange chromatography.

2. The various areas of brains were obtained from the individual dying of accident and from fetuses on the fifth and eighth gravid months. Four fresh brain tissues were obtained during the surgical operation in the patients with cerebral tumors or with epilepsy.

3. In general the distribution pattern of the free amino acids in the various areas of the human brains resembled that in dog. γ-Aminobutyric acid was detected in a large amount in the globus pallidus and the hypothalamus.

4. The quantities of free amino acids in the fetal brains differ significantly from those in the mature human brain. Taurine and phosphoethanolamine were detected in a larger amount and glutamic acid, aspartic acid, γ-aminobutyric acid and N-acetylaspartic acid were less.

5. In the brains from brain tumors there was a considerable amount of cystathionine. In the brains of accidental death and of epilepsy this sub-

stance was far less. In the fetal brains this could hardly be recognized.

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CYSTEIC ACID PEPTIDES FROM OXIDIZED TAKA-AMYLASE A

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A simple method (*1*) for the preparation of crystalline Taka-amylase A (TAA) made it possible to obtain enough amount of this amylase for extensive studies on the chemical structure, such as the determination of amino acid composition (*2*), carbohydrate components (*3*), amino- and carboxyl-terminal amino acids (*4*, *5*), amino-terminal peptides (*6*), glycopeptides (*7*) and some other peptides (*8*) of crystalline TAA. To study the relationship between the structure and the function of the enzyme protein, it is necessary to study not only the primary chemical structure of the enzyme protein but also the secondary chemical structure, especially -S·S- bridge of cystine residue. Isolation and characterization of cystine peptide from protein are very difficult, since cystine residues in protein and peptide are unstable during acid hydrolysis.

Recently, Schram, Moore and Bigwood (*9*) accomplished the method for oxidation of protein by performic acid which could convert cystine residue to cysteic acid residue which was stable during acid hydrolysis. By its strongly acidic nature, cysteic acid peptide was very easily isolated and the structure of cysteic acid peptides from oxidized ovalbumin (*10*) and papain (*11*) were determined. Therefore, the isolation of cysteic acid peptides from oxidized TAA was attempted.

Crystalline TAA was oxidized by performic acid and cysteic acid peptides were isolated by Dowex-2 column chromatography after partial hydrolysis with hydrochloric acid. Cysteic acid peptides from oxidized TAA were characterized as follows: Thr-$CySO_3H$, Pro-$CySO_3H$, Asp-$CySO_3H$, Phe-$CySO_3H$, $CySO_3H$-Glu, Tyr-[Asp, $CySO_3H$], $CySO_3H$-[Gly, Ser], Ser-[Asp, $CySO_3H$], $CySO_3H$-[Gly, Ser, Leu].

MATERIALS AND METHODS

Taka-amylase A—Crystalline TAA prepared from 'Takadiastase Sankyo' was recrystallized three times from aqueous acetone solution, as described in the previous paper (*1*). After washing with 50 per cent aqueous acetone and anhydrous acetone, the recrystallized TAA was desiccated *in vacuo*.

Performic Acid Oxidation—TAA was oxidized with the performic acid reagent essentially by the method of Schram, Moore and Bigwood (*9*). The performic acid reagent was prepared just before the experiment by mixing 40 ml. of H_2O_2 (30%) and 250 ml. of formic acid (90%) and allowed to stand for 30 minutes at room temperature. Eight gram of crystalline TAA which was dissolved in 110 ml. of formic acid (90%) was cooled in a ice-water bath and was added the preformed performic acid which had been cooled in a ice-water bath. After two hours in the ice-water bath, the reaction mixture was diluted with 400 ml. of water and evaporated *in vacuo* below 40°. Dilution with water and evaporation were repeated several times to remove the reagent.

Partial Hydrolysis of the Oxidized TAA—In the Exp. I, 1 g. of the oxidized TAA was dissolved in 20 ml. of 12 *N* HCl and in the Exp. II, 3 g. of the oxidized TAA was dissolved in 30 ml. of 12 *N* HCl and both solutions were kept for five days at 37°. Both of the hydrolysates were brought to dryness over solid NaOH in a vacuum desiccator.

Ion Exchange Column Chromatography—The hydrolysate was dissolved in a small volume of water and applied to a 2×15 cm. column of Dowex-50×8 (100–180 mesh, H^+ form) and eluted with water. A total of 100 ml. of water effluent was evaporated to dryness *in vacuo*, dissolved in a small amount of water and applied to a 2×50 cm. column of Dowex-2 (180–250 mesh, Cl^- form). Elution was made with diluted hydrochloric acid of 0.0075 *N* and 0.01 *N* in the Exp. I or by increasing its concentration from 0.0025 *N* to 0.01 *N* stepwisely in the Exp. II and collected in 5 ml. fractions. Quantity of peptide in 0.5 ml. of every fraction was estimated by the ninhydrin method of Moore and Stein (*12*).

Further Separation of Peptide by the Paper Chromatography—Each fraction of the Dowex-2 column chromatography was applied to the further separation of peptide through the paper chromatography in the descending way using the solvent mixture of *n*-butanol : glacial acetic acid : water (2 : 1 : 1 by volume). Because of the slow movement of cysteic acid peptide in this solvent, the solvent was allowed to drip off the lower edge of the paper of 30 cm. in length for as long as 15 to 20 hours. Instead of R_f value of the peptide, the ratio of the travelled distance of peptide to that of glutamic acid, which was chromatographed simultaneously, was listed in Table I. In some unsuccessful cases of the separation, peptides were chromatographed as dinitrophenylpeptides using the mixture of *n*-butanol and one per cent ammonia (1 : 1) or 1.5 *M* phosphate buffer of pH 6.0.

Amino Acid Composition of Peptide—An appropriate aliquot of purified peptide was hydrolysed with the distilled 6 *N* HCl in sealed tube for 20 hours at 110° and the resulted amino acids were identified by the paper chromatography using the solvent mixture of phenol : water (4 : 1 by weight) or of *n*-butanol : glacial acetic acid : water (4 : 1 : 1 by volume). In some cases, Levy's dinitrophenylation method (*13*) was applied further for the determination of amino acids.

Amino-Terminal Amino Acid of Peptide—The purified peptide was dinitrophenylated as described by Sanger (*14*) and DNP-peptide was hydrolysed in distilled 6 *N* HCl for 4 to 8 hours at 110°. DNP-amino acid in the ether layer was determined by two-dimentional paper chromatography of Levy (*13*) or Koch and Weidel (*15*), and the remaining amino acids in the aqueous phase by the paper chromatagraphy and Levy's dinitrophenylation method as described in the foregoing test.

RESULTS AND DISCUSSION

The chromatograms of strongly acidic peptides from the oxidized TAA on Dowex-2 column was shown in Fig. 1. The amino acid composition of purified peptide, DNP-bound and other amino acids of purified DNP-peptide were shown in Table I.

Fraction A and B in the Exp. I and fraction A, B and C in the Exp. II (Fig. 1) did not contain cysteic acid, so these may be aspartic acid, glutamic acid and acidic peptides which were composed of aspartic acid or/and glutamic acid.

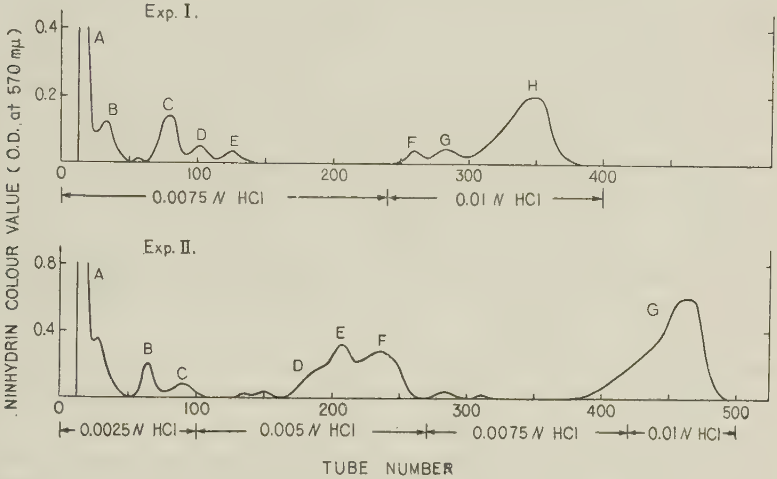

FIG. 1. Column chromatograms of the strongly acidic peptide fraction from oxidized Taka-amylase A on Dowex-2.

TABLE I

Cysteic Acid Peptides Obtained from the Oxidized Taka-Amylase A

Exp.	Fraction	R_f/R_fGlu	Amino Acid Composition	DNP-peptide		Probable Sequence
				DNP-Amino Acid	Amino Acid	
I	C_1	0.57	$CySO_3H$, Thr	Thr	$CySO_3H$	Thr-$CySO_3H$
	C_2	0.81	—	DNP-OH	$CySO_3H$, Pro	Pro-$CySO_3H$
	D_1	0.76	$CySO_3H$, Glu	$CySO_3H$	Glu	$CySO_3H$-Glu
	E_1	1.8	$CySO_3H$, Asp, Tyr	?	$CySO_3H$, Asp	Tyr-[Asp, $CySO_3H$]
	$H_2$¹⁾	—	—	Asp	$CySO_3H$	Asp-$CySO_3H$
II	D_1	0.47	$CySO_3H$, Gly, Ser	$CySO_3H$	Gly, Ser	$CySO_3H$-[Gly, Ser]
	E_1	0.56	$CySO_3H$, Thr	Thr	$CySO_3H$	Thr-$CySO_3H$
	E_2	1.2	$CySO_3H$, Gly, Ser, Leu	$CySO_3H$	Gly, Ser, Leu	$CySO_3H$-[Gly, Ser, Leu]
	F_2	0.82	$CySO_3H$, Pro	DNP-OH	$CySO_3H$, Pro	Pro-$CySO_3H$
	$G_2$¹⁾	—	—	Asp	$CySO_3H$	Asp-$CySO_3H$
	$G_3$¹⁾	—	—	Phe	$CySO_3H$	Phe-$CySO_3H$
	$G_4$¹⁾	—	—	Ser	$CySO_3H$, Asp	Ser-[Asp, $CySO_3H$]

1) Separated as DNP-peptide.

Fraction C_2 in the Exp. I and fraction F_2 in the Exp. II (Table I) produced dinitrophenol in a relatively large amount but did not produce any detectable amount of DNP-amino acid by acid hydrolysis of DNP-peptide. Based upon the result and the known fact that DNP-proline is decomposed to dinitrophenol and free proline during acid hydrolysis (*16*), N-terminal amino acid of DNP-peptide in this fraction should be assumed to be DNP-proline.

On the paper chromatogram of the hydrolysate of the fraction E_1 in the Exp. I, one gray spot was observed on the position between valine and leucine when the solvent mixture of *n*-butanol : acetic acid : water was used and between glycine and tyrosine when the solvent mixture of phenol : water was used. Sanger and Tuppy (*17*) reported that tyrosine-X from oxidized insulin moved faster than valine and slower than leucine on the paper chromatogram with the solvent mixture of *n*-butanol : acetic acid : water (4 : 1 : 5), and Thompson (*18*) reported the identity of 3-chlorotyrosine with the tyrosine-X of Sanger and Tuppy and its R_f value. In comparing the R_f value of the gray spot with that of 3-chlorotyrosine, the gray spot was assumed to be 3-chlorotyrosine.

Both fraction H in the Exp. I and fraction G in the Exp. II (Fig. 1) were mixtures of the very strongly acidic peptides and it was unsuccessful to separate peptides in the fraction by paper chromatography, therefore peptides were separated as DNP-derivatives by using the solvent mixture of *n*-butanol : 1% NH_3 or 1.5 *M* phosphate buffer of pH 6.0 as mentioned above, and the structure of three peptides were determined.

An additional amount of cysteic acid was gained after the complete hydrolysation of eluate which was obtained by the further washing of the water-washed Dowex-50 column with 1 *N* NH_3. The cysteic acid peptides containing basic amino acid would behave as neutral peptides and might be retained by Dowex-50 column as reported by Flavin and Anfinsen (*10*) and Kimmel, Thompson and Smith (*11*).

SUMMARY

A several cysteic acid peptides were separated from the partial hydrolysate of oxidized Taka-amylase A through column chromatography of Dowex-2 and the structure of the following nine peptides were determined: Thr-$CySO_3H$, Pro-$CySO_3H$, Asp-$CySO_3H$, Phe-$CySO_3H$, $CySO_3H$-Glu, Tyr-[Asp, $CySO_3H$], $CySO_3H$-[Gly, Ser], Ser-[Asp, $CySO_3H$], $CySO_3H$-[Gly, Ser, Leu].

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PHYSIOLOGICAL STUDY ON THE OXYGEN- AND NITRATE-RESPIRATION OF PSEUDOMONAS AERUGINOSA

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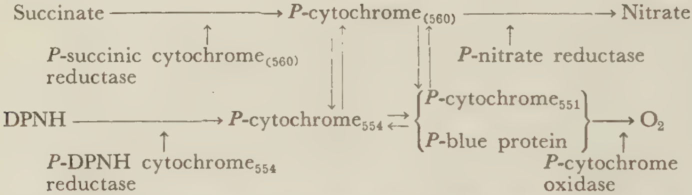
During previous work in our laboratory (*1–4*), *Pseudomonas* (*P*-) cytochrome oxidase containing the so-called cytochrome a_2, *P*-cytochrome$_{551}$, *P*-cytochrome$_{554}$ and *P*-blue protein of *Pseudomonas aeruginosa* were separated from each other and purified in a water-soluble form. Studies were made on the separate purified respiratory components and more organized cellular preparations (whole cells, cell-free extracts and the cellular fragments). From these studies, the terminal electron-transferring system of *P. aeruginosa* was depicted as follows (*2, 5*):



In addition to these components, there was an enzyme which oxidize various chemical reductants such as hydroquinone without being inhibited by cyanide. This enzyme was tentatively named *P*-hydroquinone oxidase. It was distinct from *P*-cytochrome oxidase which oxidized hydroquinone, *P*-cytochrome$_{551}$ and *P*-blue protein, but unlike *P*-hydroquinone oxidase, was strongly inhibited by cyanide and carbon monoxide. The substrate-specificity of *P*-hydroquinone oxidase to other chemical reductants differed greatly from that of *P*-cytochrome oxidase (*6*). The true function of *P*-hydroquinone oxidase in living cells has not yet been ascertained.

In order to clarify the physiological interrelationship of oxygen- and nitrate-respiration of the organism, this paper deals with some aspects of variations in the mode of respiration under various growth conditions.

MATERIALS AND METHODS

Bacterial Strain and Culture Methods—The same strain of *Pseudomonas aeruginosa* was used throughout this series of experiments (*1–6*). The stock culture was grown on bouillon peptone agar. For experiments, cells were grown at 37° for 20 hours in a medium containing per liter of water following: bouillon, 10 g.; peptone, 10 g.; KH_2PO_4, 6.4 g.; Na_2HPO_4, 3.6 g.; pH was adjusted to 6.5 (liquid standard medium). In some experiments, 20 g. of KNO_3 was added to the medium (liquid nitrate medium). In other experiments, the medium was solidified by adding 3 per cent agar (solid standard medium and solid nitrate medium).

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Preparation of Acetone Powder of the Cells—After washings three times with distilled water, the cells were treated with cold acetone (0°-5°) for 10 minutes as described in the previous report (*1*).

Preparation of Cell-Free Extract—After washing three times with distilled water the cells were ground with two volumes of quartz sand for approximately one hour in an ice bath. The resulting cellular paste was extracted overnight with an equal volume of distilled water in a refrigerator. It was then centrifuged at 14,500×*g* for 15 minutes. The supernatant was used as the cell-free extract.

Measurement of Gas Evolution and Consumption—Measurements were made in a Warburg manometer. After temperature equilibration at 30° for about 10 minutes, reactions were started by adding the substrate.

Estimation of Cytochrome Components—A microspectroscope was used. *P*-cytochrome oxidase and *P*-hydroquinone oxidase were measured by their abilities to oxidize hydroquinone in the presence and absence of cyanide.

RESULTS AND DISCUSSION

Difference in Content of P-Cytochrome Oxidase and P-Hydroquinone Oxidase in Aerated and Submerged Cultures—Activity to oxidize hydroquinone could not be assayed in whole cells, perhaps because of lack of *cellular permeability* so that hydroquinone could not reach the hydroquinone-oxidizing enzymes. Hydroquinone inhibited endogenous cellular respiration. An extract from the acetone powder of the cells was therefore used to compare the activity of cells grown in aerated and submerged cultures. Pyocyanine, produced by aerated but not by submerged cultures, was removed by acetone treatment. Extracts of acetone powders of cells grown in liquid standard medium with vigorous aeration, and in liquid nitrate medium in submerged cultures both oxidized hydroquinone (Fig. 1).

The submerged culture extract oxidized hydroquinone twice as rapidly as the aerated culture extract in the absence of cyanide. However in the presence of $10^{-3}\,M$ potassium cyanide, the activity of the cells grown by submerged culture was more than 70 per cent inhibited, while that of the aerated cells was hardly affected. The oxidation of hydroquinone by *P*-cytochrome oxidase has been found to be completely inhibited by this concentration of cyanide, but *P*-hydroquinone oxidase is not affected (*6*). Therefore cells grown by aerated culture have much less *P*-cytochrome oxidase than those from submerged culture. The oxidation of hydroquinone by cells from submerged culture was more than 70 per cent due to *P*-cytochrome oxidase and the rest to *P*-hydroquinone oxidase, if all of the cyanide-sensitive activity can be attributed to *P*-cytochrome oxidase and the cyanide-insensitive to *P*-hydroquinone oxidase. If this is so there is 40 per cent more *P*-hydroquinone oxidase in aerated than in submerged cells. With a microspectroscope, whole cells show an absorption band of so-called cytochrome a_1 around 590 mμ (*1*). This band was lost after acetone treatment. Attempts to extract *P*-cytochrome oxidase from the acetone powder of aerated cells by the standard method used for submerged cells (*3*) were unsuccessful. This negative data supports the idea that cells grown by aerated culture do not contain a significant amount of *P*-cytochrome oxidase.

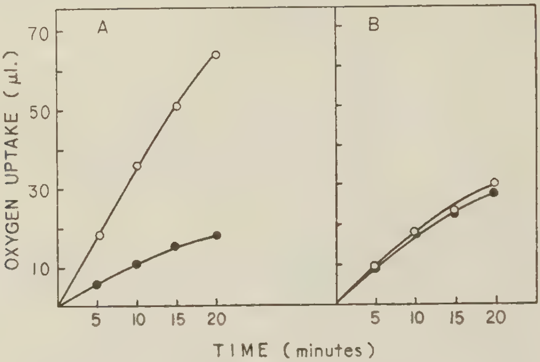

FIG. 1. Hydroquinone oxidation by acetone powder extract of *Pseudomonas aeruginosa.*

A: Cells grown in liquid nitrate medium.

B: Cells grown in liquid standard medium with vigorous aeration.

Reaction mixture contained: enzyme solution, 1.2 ml.; *M*/5 phosphate buffer (pH 6.6), 0.4 ml.; 10^{-2} *M* KCN (—●—) or distilled water (—○—), 0.2 ml.; *M*/2 hydroquinone, 0.2 ml. Reactions at 30° under air.

Quantitative Difference in Cytochrome Components in Aerated and Submerged Cultures—Cells grown by submerged culture had more *P*-cytochrome$_{551}$ and slightly less *P*-cytochrome$_{554}$ than those grown by aerated culture. Moreover, more *P*-cytochrome$_{554}$ was produced when cells were grown in a medium supplemented with one per cent succinate or lactate. The submerged cells had slightly more *P*-cytochrome$_{(560)}$ than the aerated cells (Table I). It was difficult to estimate the content of *P*-blue protein, due to its diffused and broad absorption spectrum around 630 mμ (*2*). These quantitative variations in the individual respiratory components under different culturing conditions may result from differences in the role of each component in the aerobic and anaerobic respiration of the microorganism. Lenhoff *et al.* (*7*), and Rosenberger and Kugot (*8*) indicated that lowered oxygen tension favoured production of cytochrome components in pseudomonads. This is consistent with results on *P*-cytochrome$_{551}$ and *P*-cytochrome oxidase, but not *P*-cytochrome$_{554}$.

Influence of Cyanide on Endogenous Cellular Respiration in Aerobic and Submerged Cultures—During experiments with acetone powder of the cells, the pyocyanine could be extracted by treatment with acetone. Thus the participation of pyocyanine could be excluded. In experiments with whole cells, it is impossible to remove pyocyanine completely from the cells simply by washing, since the pigment is more or less produced under aerobic conditions and since it seems to be partially bound to the respiratory fragments of the cells. In the following experiment, cells were grown on the solid standard medium. On this medium they contained much less pyocyanine than when grown in the liquid medium with aeration.

TABLE I

Relative Quantities of Terminal Respiratory Components in Pseudomonas aeruginosa Grown by Aerated and Submerged Culture

Respiratory Component	Relative Quantity	
	Culture	
	Aerated	Submerged
P-Cytochrome$_{551}$	++	++++
P-Cytochrome$_{554}$	++ (if succinate or lactate added +++)	+
P-Cytochrome$_{(560)}$	++	+++
P-Blue protein	(hard to estimate)	
P-Cytochrome oxidase	+	++++
P-Hydroquinone oxidase	++	+

The endogenous respiration of cells grown aerobically was about twice as high as that of cells grown in the liquid nitrate medium by submerged culture (Fig. 2). Potassium cyanide (10^{-3} M) did not influence the endogenous respiration of the aerobic cells, but inhibited nearly 50 per cent of that of cells grown by submerged culture. This agrees with the results shown in Fig. 1.

Addition of nitrate to the culture medium inhibited the production of pyocyanine very much in aerobic cultures and completely in submerged cultures. When the aerobic cells were aerated, the pyocyanine in the cells changed from pink to blue. When the aeration was discontinued, the pink-colour was gradually restored. These phenomena could also be observed in a particulate fraction obtained from the aerobic cells. This indicates that the pigment has indeed some role in the oxygen respiration. It is still unknown in what way the pigment was reduced in the cell, or if related with *P*-hydroquinone oxidase. Results with aerobic cells are inevitably confused by the presence of pyocyanine.

Respiration of Cells Grown under Various Culture Conditions; at Different Oxygen Tensions and in the Presence and Absence of Nitrate—Aerobic growth of cells was inhibited by the presence of nitrate (Table II). Cells grown under different conditions could oxidize lactate in roughly the same rate. However, cells grown at a low oxygen tension oxidized succinate at a notably slow rate

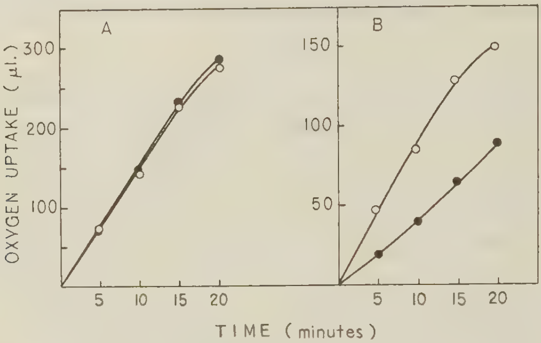

FIG. 2. Influence of cyanide on endogenous respiration of *Pseudomonas aeruginosa.*

A : Bacterial cells grown in solid standard medium.

B : Bacterial cells grown in liquid nitrate medium.

Reaction mixture contained : Cell suspension, 1.0 ml. ; $M/5$ phosphate buffer (pH 7.2), 0.4 ml. ; 10^{-2} M KCN (—●—) or distilled water (—○—), 0.2 ml., total volume adjusted to 2.0 ml. with distilled water. 0.2 ml. of 20% KOH was placed in the center well. Reactions at 30° under air.

TABLE II

Growth and Respiration of Pseudomonas aeruginosa Cultured under Various Conditions

Solid culture		Total dry wt. of the cells (Relative val. after 20 hrs.)	Respiration (XO_2μl./10 mg./hr.)				R.Q. +glucose
Gas phase	KNO_3		endo.	+lactate	+ succinate	+ glucose	
N_2	+	100	265	312	31	21	1.0
air	+	94	358	491	31	21	0.3
air	—	198	436	389	62	190	0.4
O_2	+	111	374	257	93	44	0.5
O_2	—	146	249	405	117	85	0.3

Experimental conditions were almost the same as those in Fig. 2. Reactions were started by mixing the 2 μM of substrate with the bacterial suspension (4 mg./ml.) buffered at pH 6.6 at 30° under atmospheric conditions. When substrate was added, respiration was calculated from (XO_2 in the presence of substrate)-(endogenous XO_2).

than cells grown at a high oxygen tension. As shown in Table I, aerated cultures formed more *P*-cytochrome$_{554}$ and less *P*-cytochrome$_{(560)}$. The relationship between these facts is still unknown if consideration is going to the scheme of the cytochrome system proposed by Yamanaka (*5*).

When anaerobically grown on the nitrate medium, the cells oxidized glucose with an R. Q. of nearly one, which was very different from the R. Q. of 0.3–0.4 of cells grown under the other conditions in Table II. It seems likely that glucose was oxidized in a different manner in each case. Campbell *et al.* reported that the pathway of glucose degradation in *Pseudomonsa aeruginosa* was as follows in cells grown aerobically: Glucose→Gluconate→2-Ketogluconate. From other experiments, they suggested that there was no Embden-Meyerhof route (*9*, *10*).

Gas Production of Bacterial Cells in the Presence of Nitrate—As is well known, *Pseudomonas aeruginosa* is a facultatively anaerobe, and it can grow anaerobically if nitrate is present in the medium. It liberates much gas, mainly nitrogen and carbon dioxide. The nitrate reduction of cells grown under various conditions was therefore compared by measuring gas evolution. As shown in Table III, much gas was produced by cells grown in the presence of nitrate under anaerobic and atmospheric conditions. On the other hand, no gas was formed by cells grown in the absence of nitrate under atmospheric conditions or at a high oxygen tension in the presence of nitrate. However under all conditions mentioned above, the cells reduced a fair amount of nitrate to nitrite (cf. *5*). Therefore the system which reduces nitrate to nitrite at least is a constitutive enzyme(s) and the system which reduces the enzymatic intermediate further to gases is formed adaptively. Even under conditions where no gas was formed, the growth of cells (measured by the dry weight) was reduced in the presence of nitrate, as shown in Table II. This shows that nitrate or the intermediate formed by the constitutive nitrate-reducing enzyme(s) of the cells inhibits cell-growth. A similar strong inhibition was observed when cells were grown in the presence of nitrate under atmospheric conditions.

TABLE III

Gas Production of Pseudomonas aeruginosa in the Presence of Nitrate

Solid culture		Gas production (μl./10 mg./hr.)		Inhibition (%)			
				KCN (5×10^{-3} *M*)		CO (100%)	
Gas phase	KNO_3	endo.	+ lactate	endo.	+ lactate	endo.	+ lactate
N_2	+	156	390	90	100	38	17
air	+	23	187				
air	—	0	39				
O_2	+	0	16				
O_2	—	0	15				

Reaction mixture contained: Bacterial suspension (8 mg./ml.), 1.0 ml.; *M*/5 phosphate buffer (pH 6.6), 0.4 ml.; 5×10^{-2} *M* KCN or distilled water, 0.2 ml.; *M*/2 KNO_3, 0.1 ml. *M*/100 sodium lactate or distilled water, 0.2 ml., total volume adjusted to 2.0 ml. with distilled water. 0.2 ml. of 20% KOH was placed in the center well. Reactions at 30° under nitrogen, except with CO. The latter reaction took place in the dark.

As with oxygen respiration cyanide inhibited gas production by the cells. However in contrast to the cytochrome oxidase system, gas production was unaffected by carbon monoxide.

Comparison of Oxygen-Uptake and Gas Production by Cells with Various Substrates—Cells grown anaerobically in the presence of nitrate consumed oxygen, and in anaerobic conditions produced gas in the presence of nitrate, if various substrates were added (Table IV). The various substrates tested affected oxygen uptake and gas production similarly. This physiological similarity suggests that the oxygen respiration including the cytochrome oxidase system and the nitrate reducing system may be mutually interrelated. In other experiments, Yamanaka showed that *P*-cytochrome$_{(560)}$ and *P*-cytochrome$_{554}$ play important direct roles in both systems (*5*).

TABLE IV

Oxygen Uptake and Gas Production of Pseudomonas aeruginosa with Various Substrates

Substrate	Relative activity	
	Oxygen uptake	Gas production
Lactate	100	100
(endogenous respiration)	67	30
Malate	30	25
Acetate	26	18
Succinate	18	15
Citrate	9	3
Glucose	8	2

Reaction mixture contained: Bacterial suspension (8 mg./ml.), 1.0 ml.; *M*/5 phosphate buffer (pH 6.6), 0.4 ml.; *M*/100 substrate, 0.2 ml.; when estimating gas production, *M*/2 KNO_3, 0.1 ml., total volume adjusted to 2.0 ml. Reactions at 30°. The gas phase was air for estimation of oxygen consumption and nitrogen for measuring gas production.

Oxidation of Hydroquinone and Lactate by Cell-Free Extracts from Cells Grown in the Presence of Nitrate under Anaerobic Conditions and by Those from Cells Grown in the Absence of Nitrate under Atmospheric Conditions—The oxidation of hydroquinone and lactate was compared in cell-free extracts of aerobic and anaerobic cultures (Table V). The results on hydroquinone oxidation agree with those with the acetone powder of the cells (Fig. 1). The lactate oxidizing activity showed a similar tendency to that of hydroquinone oxidation, but was more sensitive to cyanide. Since the concentration of cyanide used was high enough to inhibit the *P*-cytochrome oxidase completely, the endogenous oxidation and the lactate oxidation which were not inhibited by cyanide might be catalyzed by an oxidative enzyme(s) other than *P*-cytochrome oxidase. The cyanide-insensitive activities of cells grown aerobically and anaerobically were very different. These differences in cyanide sensitivity

TABLE V

Oxidation of Hydroquinone and Lactate by Cell-Free Extracts from Pseudomonas aeruginosa

Solid culture		Oxidation (O_2 uptake μl./10 mg./hr.)			
		+Hydroquinone		+Lactate	
Gas phase	KNO_3		KCN inhibition (%)		KCN inhibition (%)
N_2	+	96	50	20	80
air	−	80	30	52	69

Reaction mixture contained: Cell-free extract, 1.2 ml.; *M*/5 phosphate buffer (pH 6.6), 0.4 ml.; 5×10^{-2} *M* KCN or distilled water, 0.2 ml.; *M*/2 substrate, 0.2 ml., total volume adjusted to 2.0 ml. with distilled water. 0.2 ml. of 20% KOH was placed in the center well. Reactions at 30° under atmospheric conditions.

may show that cyanide-insensitive terminal oxidative enzymes such as *P*-hydroquinone oxidase have a true physiological function.

SUMMARY

Respiratory systems are greatly affected by the physiological conditions of the cell. Oxygen and nitrate as terminal electron acceptors of biological oxidation regulate the bacterial (*Pseudomonas aeruginosa*) respiratory chains, qualitatively and quantitatively. When the environmental conditions are altered, the concentration of each cytochrome component fluctuates according to its situation in the system. The oxygen respiration system, that is, the cyanide-sensitive and -insensitive, and nitrate-reducing systems were studied in this respect. Possible roles of *P*-cytochrome oxidase and *P*-hydroquinone oxidase in the terminal electron transport are discussed.

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The author would like to express his thanks to Prof. K. Okunuki and Dr. T. Horio for their valuable guidance. Thanks are also due to his colleagues Messrs. M. Nozaki, T. Yamanaka, J. Yamashita and H. Mizushima of the same laboratory for their help and discussion and Messrs. M. Nakai, K. Yamazaki and K. Kusai of Amagasaki Factory of Nagase & Co., Ltd., for massculture of the bacteria.

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STUDIES ON THE DISTRIBUTION AND PHOSPHATE-TURNOVER OF THE ACID-SOLUBLE PHOSPHORUS COMPOUNDS IN VARIOUS NORMAL AND NEOPLASTIC TISSUES OF RATS

I. A SEMI-MICRO GRADIENT ELUTION CHROMATOGRAPHY WITH DOWEX 1 FORMATE AND IDENTIFICATION OF SOME PHOSPHATE ESTERS ON THE CHROMATOGRAM

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Since the elaboration of the gradient elution chromatography of acid-soluble nucleotides by Hurlbert, Schmitz, Brumm, and Potter in 1954 (*1*), many analytical studies have been reported from their (*2–6*) and other laboratories (*7–9*) with the use of this method. This method is, not only valuable for the analysis of acid-soluble phosphorus compounds, but also applicable to other tracer experiments, and will be able to play a significant role for elucidating tissue metabolic patterns. Thus, along this line, more extensive studies are now being desired.

The authors intended to analyse acid-soluble fractions from various rat tissues including tumors, and to compare the distribution specificities and metabolic patterns of various detectable compounds in these tissues. Although the above mentioned technique showes excellent resolving ability, it is not always convenient for analysing small amounts of specimen. So a modified semi-micro method suitable for analysing 1 to 3 g. of tissue was worked out and applied to rat tissues. Radioactive P^{32} was also injected to the animals and the distribution of the radioactivity in the liver acid-soluble fraction was determined with this modified semi-micro method.

During the course of these experiments, four radioactive compounds, which were hitherto unidentified on the chromatogram, were isolated and three of them were identified as glucose-6-phosphate, L-α-glycerophosphate, and a phosphorus compound containing amino acids.

The details of these experiments will be reported below.

METHODS

Preparation of Perchloric Acid Extracts—Two pairs of the rats of Wistar-King A strain were supplied from the National Institute of Genetics* (Litter No. WKA/MS, F152), and the inbred offspring of them grown in this laboratory were used throughout the experiments. The foods and water were given *ad libitum*.

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Animals weighed 100 to 120 g. were decapitated, and the tissue was removed immediately and frozen in acetone-dry ice. This was carried out as fast as possible, usually in 1 to 2 minutes. 1 to 3 g. of the frozen tissue was crushed in an ice cold porcelain mortar with a small amount of dry ice. The crushed pieces were immediately homogenized with two volumes of ice cold 0.6 *N* perchloric acid in a glass homogenizer. During these treatments, any local melting was avoided as far as possible. The homogenate was centrifuged and the supernatant was removed. The precipitate was washed once with two volumes of 0.2 *N* perchloric acid by centrifuging. The supernatant and the washing were combined, added with a few drops of 0.2 per cent phenol red solution, cooled in ice-water, and then cautiously neutralized first with 30 per cent KOH and then with 1 *N* KOH. The resulting precipitate was centrifuged, and the supernatant was stored in a refrigerator for two hours. The small amount of precipitate formed after 2 hours was separated by centrifuging or decantation, and the final supernatant was immediately applied to the column chromatography.

Preparation of the Dowex 1 Formate Column—Dowex 1 chloride resin (X10, 200–400 mesh) was repeatedly treated with 3 *M* sodium formate until chloride ion could scarcely be detected in the washings. During these treatments, too fine particles of the resin were removed by decantation as far as possible. The resulting Dowex 1 formate resin was washed with water and kept in a refrigerator until use. The washed resin was poured little by little into a 0.6×50 cm. column with a stop-cock at one end and filled with water. The length of the resin column was set to 40 cm. The resin column was washed with 250 ml. of the mixture of 1 *M* sodium formate and 4 *N* formic acid (1 : 1), and then with 150 ml. of conc. formic acid. Finally, 500 to 600 ml. of water was passed through the resin column until the effluent showed blue color with bromphenol blue test paper (pH above 4). These conditionings of the resin column consumed 3 to 4 days.

Gradient Elution—After the acid soluble extract was adsorbed on the resin column, the gradient elution was carried out under following conditions :— a) mixer : 150 ml. of water, b) flow rate of the eluting solvent : 8 to 12 ml. per hour, c) collection of the eluting fraction : each 4 ml., d) total fractions collected : 200 tubes, e) time consumed : 3 to 4 days continuously.

Solutions in the reservoir were changed in the following way.

Tube No.	Solution in the Reservoir
1– 5	water
6–15	1 *N* formic acid
16–60	4 *N* formic acid
61–110 (61–105)	0.2 *M* ammonium formate in 4 *N* formic acid
111–130 (106-125)	0.4 *M* ammonium formate in 4 *N* formic acid
131–180 (126-180)	0.8 *M* ammonium formate in 4 *N* formic acid
180–200	2 *M* ammonium formate in 4 *N* formic acid

Preparation of the Dowex 50 Column—Dowex 50 (X4, 200-400 mesh) resin was repeatedly treated with 4 *N* HCl, and then washed with large volumes of water until the washings showed no acidic reaction. The resin column was prepared in almost the same way as described in the case of Dowex 1 resin.

Determination of the Ultraviolet Absorption—The U. V. absorption of the fractions obtained by the chromatography was directly measured at the wavelength of both 260 and 275 mμ with a Hitachi Spectrophotometer (type EPU-2)*. No dilution of the samples with dil. HCl was carried out for U. V. absorption measurements except for cases which showed too high absorption values. For each of successive 9 fractions, a blank solution for the photometry was prepared. The concentration of ammonium formate and formic acid to be contained in these blanks was calculated by the equation described by Hurlbert *et al.* (*1*).

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* Hitachi, Ltd., Tokyo, Japan

Measurement of Radioactivity—Sixty μc of P^{32} (in about 1 ml.) was injected into the peritoneal cavity of a rat. 2 mg. of KH_2PO_4 was added per ml. of P^{32} solution as a carrier. Each fraction obtained from the chromatography with Dowex 1 was made up to 24 ml. by adding dil. HCl, and the radioactivity was measured with a liquid counter for hard β-rays (supplied from Kōbe Kōgyo Co. Ltd*., GM-413). The radioactivity of the fractions obtained from the chromatography with Dowex 50 was measured in a usual way using counting plates. All counts determined were corrected for the decay of P^{32} (*10*).

Paper Chromatography—The Toyo Filter Paper** No. 53 was used. The solvent mixtures chiefly employed were as follows:

a) Methanol-Formic Acid-Water (80 : 15 : 5) (*11*)
  80 volumes of methanol, 15 volumes of concentrated (85 per cent) formic acid, and 5 volumes of water.
b) Methanol-Ammonia-Water (60 : 10 : 30) (*11*)
  60 volumes of methanol, 10 volumes of concentrated (28 per cent) ammonia, and 30 volumes of water.
c) *tert*-Butanol-Picric Acid-Water (80 : 2 : 20) (*12*)
  80 ml. of *tert*-butanol, 2 g. of picric acid, and 20 ml. of water.
d) *n*-Butyric Acid-NaOH-Water (69 : 0.85 : 31) (*13*)
  0.85 g. of NaOH was dissolved in 100 ml. of 69 per cent (v/v) *n*-butyric acid.
e) Phenol-Water (72 : 28) (*14*)
f) *n*-Butanol-Propionic Acid-Water (10 : 5 : 7) (*14*)
g) Ethanol-Acetate (pH 3.5) (*15*)
  80 per cent ethanol containing 0.8 per cent acetate at pH 3.5
h) Ethanol-Acetate (pH 4.8)
i) *n*-Butanol-Pyridine (4 : 1) (*16*)
j) *n*-Butanol-Acetic Acid-Water (4 : 1 : 1)
  4 volumes of *n*-butanol, one volume of glacial acetic acid, and one volume of water.
k) *n*-Butanol-Acetic Acid-Water (100 : 30 : 85) (*17*)
l) Ethanol-Ammonia (95 : 5) (*17*)
  95 volumes of 95 per cent ethanol and 5 volumes of 28 per cent ammonia.

For the detection of phosphorus compounds on the paper, Hanes and Isherwood's molybdate reagent (*12*) was employed. After spraying this reagent, the paper was dried by air stream, and the resulting phosphomolybdate was reduced by illuminating with low pressure mercury arc. For the detection of U. V. absorbing materials on the paper, mercury arc with a liquid filter (*18*) was employed. Sugars were detected with *m*-phenylenediamine reagent (*19*). Amino acids were detected with 0.1 per cent ninhydrin in *n*-butanol. A drop of 1 *N* KOH was added to 10 ml. of the ninhydrin solution. For the detection of free choline, the Levine-Chargaff's reagent was used (*16*).

Enzymatic Estimation of L-α-*Glycerophosphate*—Partially purified L-α-glycerophosphate dehydrogenase was prepared from rabbit muscle following the method of Baranowski (*20*). Using this enzyme preparation, L-α-glycerophosphate was estimated by a modification of the method of Bublitz and Kennedy (*21*) in the presence of hydrazine as a trapping agent for dihydroxyacetone phosphate.

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Determination of Inorganic Phosphate and Pentose—Inorganic phosphate was determined by the method of Gomori (*22*), and pentose was estimated by the orcinol reaction as modified by Dische (*23*).

Preparation and Analysis of Dinitrophenyl Amino Acids—The method of Koch and Weidel (*24*) was applied for preparation and analysis of dinitrophenyl amino acids.

RESULTS

As shown in Fig. 1, some 20 peaks could be obtained from the acid-soluble extract of liver with the application of the modified semi-micro method. Although the individual peaks were not identified with the re-chromatography or paper chromatography, the main peaks could be recognized by the comparison with the results of the standard macro method reported by Hurlbert *et al.* (*1*) and Brumn *et al.* (*4*), mainly by comparing their intensities of U. V. absorption, the concentrations of the eluates at which they appeared, and the ratios of E_{275} to E_{260} which they showed. By comparing these values calculated on the semi-micro chromatogram with those reported with the use of the macro methods (*1*, *4*), it was revealed that the sequence of peaks obtained by the seme-micro analysis agreed well with that of the macro methods. The positions of adenosine phosphates on the semi-micro chromatogram were also checked using the authentic samples.

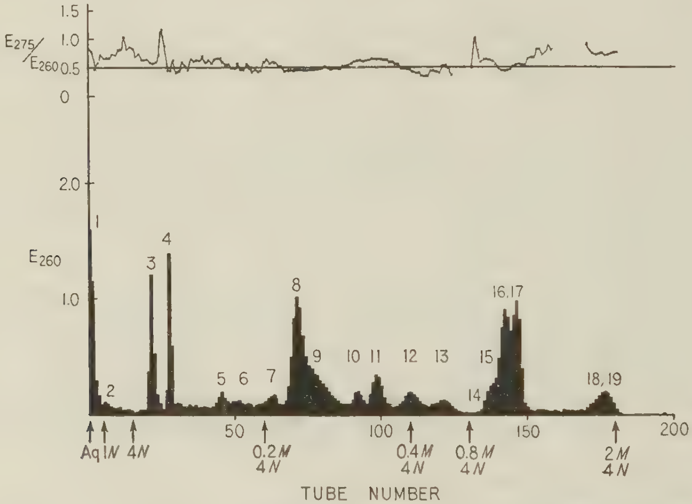


FIG. 1. Analysis of acid-soluble extract of rat liver with the use of modified semi-micro gradient elution chromatography. (Wet weight of the rat liver tissue used: 2 g.)

The main components presumed to be in the peaks are as follows:

Peak	3	DPN
	4	AMP

5	GMP
6	CDP, IMP
7	UMP
8	ADP
10	UDPAG (UDP-acetyl glucosamine)
11	UDPG (UDP-glucose)
12	ADPX
15	UDPGA (UDP-glucuronic acid)
16, 17	UDP, ATP
18, 19	GTP, UTP

The ratios of E_{275} to E_{260} are high between peaks 3 and 4, and also at position 14. These indicate the presence of CMP and CTP, respectively.

Fig. 2 illustrates a chromatogram of the acid-soluble extract of rat striated muscle. The muscle tissue was cut off from the back (the both side of the back bone) of a rat. The predominance of adenosine phosphates are seen.

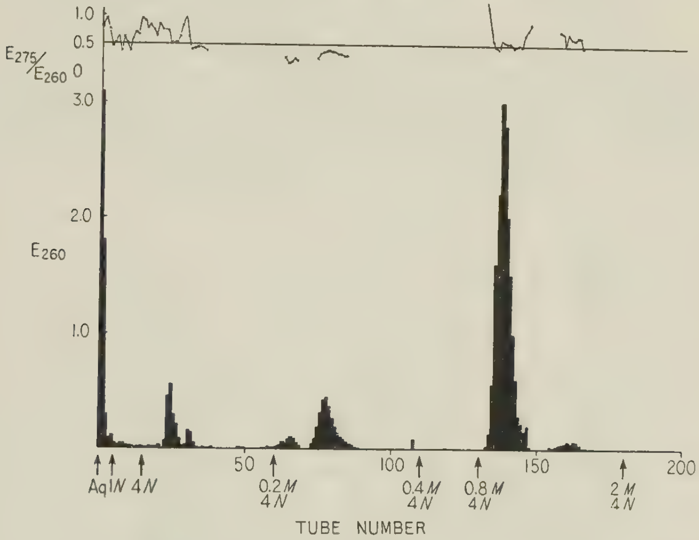


FIG. 2. Chromatography of the acid-soluble extract of rat striated muscle. (Wet weight: 1.75 g.)

Fig. 3 shows a case of rat thymus. The amounts of GTP and TUP are large in relative to that of ATP.

A precise comparison of the nucleotide distribution patterns of various tissues will be reported later.

Fig. 4 showes the distribution and phosphate turnover of acid-soluble phosphorus compounds in liver. This figure almost agrees with originally reported one (*4*).

In Fig. 4, there are four radioactive peaks which have not been hitherto

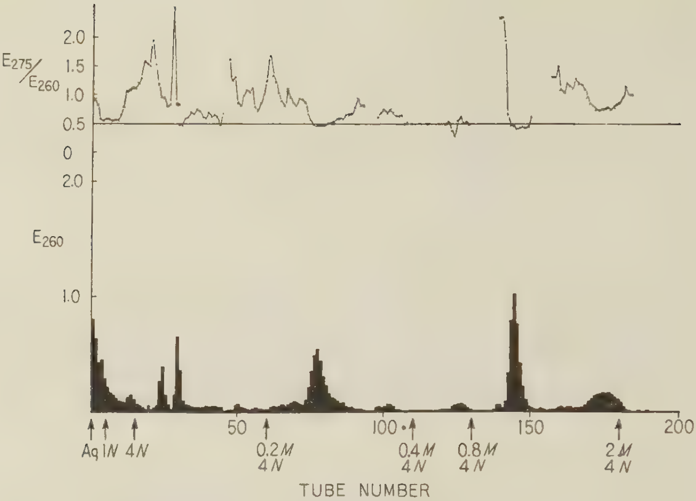


FIG. 3. Chromatography of the acid-soluble extract of rat thymus. (Wet weight of the thymus tissue: 1.5 g. The tissue were collected from 6 rats of 70 days of age.)

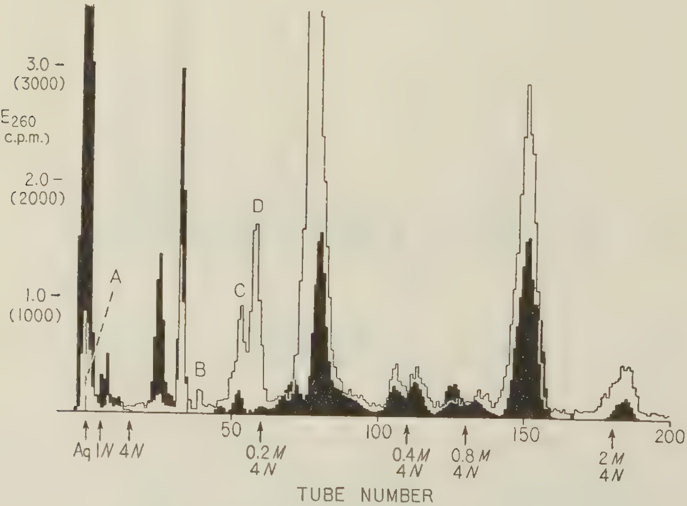


FIG. 4. Distribution and phosphate-turnover of acid-soluble phosphorus compounds in liver. (Wet weight: 2.7 g.)

The animal was sacrificed one hour after the intraperitoneal injection of P^{32}.

black peaks: U. V. absorption, colorless peaks: radioactivity. The highest peak of radioactivity is mainly that of inorganic phosphate.

identified. Peaks A is the fraction which is unadsorbed on Dowex 1 formate resin. Peak B was not precisely studied but composed from at least two components both of which showed positive spot test with ninhydrin. Peak C has been reported to be hexose-monophosphate (*4*). Peak D is well separated by this semi-micro method and, as to this, no description could be found in the literature. So, the isolation and identification of these substances were carried out.

With repeated adsorption on Dowex 1 formate column and elution by ammonium formate or formic acid, the Fraction C and D were isolated and purified (Scheme 1). After the purifications, each of them showed only one spot on the several kinds of paper chromatogram. Although Fraction D showed a positive reaction with a sensitive orcinol test for pentose, the color intensity calculated on the basis of phosphate was 30 times less than that of ribose-5-phosphate. Thus, the purified Fraction D was considered to contain small amounts of pentose phosphate but its major substance was presumed to be other than pentose phosphates.

SCHEME 1

Purification of Fr. C and D

Rat Liver PCA Extract (obtained from 40 g. of the Tissue, 5 Rats)
↓
1st Adsorption on Dowex 1 Formate
↓
Elution (gradient to 4 *N* Formic Acid)
↓
Crude Fr. (C+D)
↓
Lyophilization
↓
2nd Adsorption on Dowex 1 Formate
↓
Elution (gradient to 0.4 *M* NH_4-formate)
↓
Treatment with Amberlite IR 120
↓
Dilution with Water
↓
3rd Adsorption on Dowex 1 Formate (freshly prepared, 0.6×40 cm.)
↓
Elution (gradient to 4 *N* Formic Acid)

↓
Crude Fr. C
↓
Lyophilization
↓
4th Adsorption on Dowex 1 Formate
↓
Elution (gradient to 4*N* Formic Acid)
↓
Lyophilization
↓
Purified Fr. C
↓
Analysis

↓
Crude Fr. D
↓
,,
↓
,,
↓
,,
↓
,,
↓
Purified Fr. D
↓
Analysis

The utilized methods of analysis of Fraction C are illustrated in Table I. The Fraction C showed the identical behaviour with the authentic samples of glucose-6-phosphate in all of these examinations. Thus it was concluded that the major component of the Peak C was glucose-6-phosphate.

TABLE I

Analysis of Fraction C

Paper Chromatography (detected with Molybdate Reagent)
1) Methanol-NH_3-Water (60 : conc. NH_3 10 : 30)
2) Methanol-Formic Acid-Water (80 : 15 : 5)
3) *tert*-Butanol-Picric Acid-Water (80 : 2 : 20)
4) Phenol-Water (72 : 28)
5) Ethanol-Acetate (0.8 per cent Acetate in 80 per cent Ethanol, pH 4.8)
6) *n*-Butyric Acid-NaOH-Water (69 : 0.85 : 31)

Paper Electrophoresis
1) 9.2 per cent Butyric Acid-0.2 per cent NaOH-Water (pH 3), 1 mA/cm, 300-200 V, 8 hours

Hydrolysis with 1 N HCl at 100°
after 2 hours, *i*P liberation about 10 per cent

Orcinol Color Reaction Spectrum

Paper Chromatography of the Hydrolysis Product
hydrolyzed with 1.5 *N* HCl for 4 hours at 100°.
detected with *m*-Phenylenediamine Reagent.
1) Phenol-Water (72 : 28)
2) *n*-Butanol-Acetic Acid-Water (4 : 1 : 1)

TABLE II

Analysis of Fraction D

Paper Chromatography (detected with Molybdate Reagent)
1) Methanol-NH_3-Water (60:conc. NH 10 : 30)
2) Methanol-Formic Acid-Water (80 : 15 : 5)
3) *tert*-Butanol-Picric Acid-Water (80 : 2 : 20)
4) Phenol-Water (72 : 28)
5) Ethanol-Acetate (0.8 per cent Acetate in 80 per cent Ethanol, pH 4.8)
6) *n*-Butanol-Pyridine (4 : 1)
7) *n*-Butanol-Propionic Acid-Water (10 : 5 : 7)
8) *n*-Butyric Acid-NaOH-Water (69 : 0.85 : 31)

Papar Electrophoresis
1) 9.2 per cent Butyric Acid-0.2 per cent NaOH-Water (pH 3) 1 mA/cm, 300–200 V, 8 hours
2) *M*/60 Acetate (pH 3) 0.3–0.45 mA/cm, 600 V, 55 minutes

Hydrolysis with 1 N HCl at 100°
2 hours ; *i*P liberation less than 3 per cent

Oxidation with L-α-Glycerophosphate Dehydrogenase

With several preliminary experiments, the Fraction D was presumed to

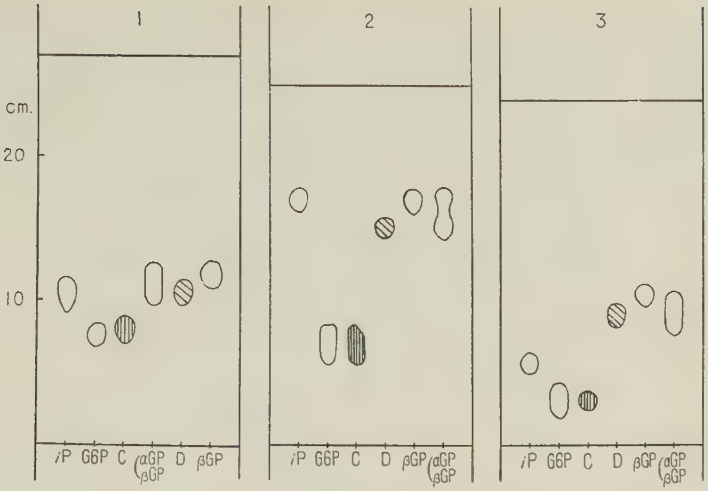


FIG. 5. Paper chromatography of fractions C and D.
(1) *n*-Butyric Acid-NaOH-Water (69 : 0.85 : 31), 24 hours at room temperature.
(2) *tert*-Butanol-Picric Acid-Water (80 : 2 : 20), 40 hours at room temperature.
(3) Phenol-Water (72 : 28), 18 hours at room temperature.
*i*P : inorganic phosphate, α-GP : α-glycerophosphate, β-GP : β-glycerophosphate

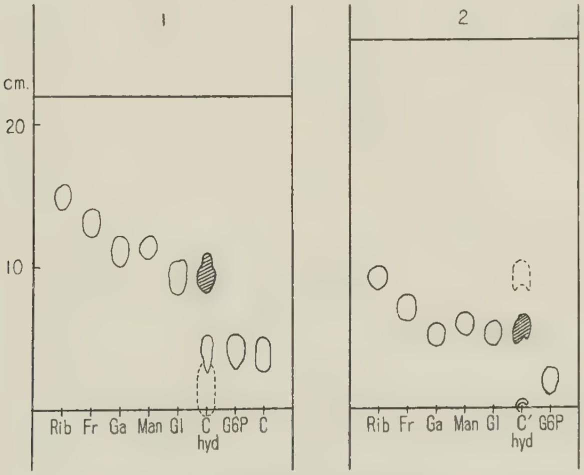


FIG. 6. Paper chromatography of fraction C after hydrolysis.
hydrolysis : 1.5 *N* HCl, 100°, for 4 hours
(1) Phenol-Water (72 : 28), 15 hours at room temperature.
(2) *n*-Butanol-Acetic Acid-Water (4 : 1 : 1), 15 hours at room temperature.

detection : *m*-phenylenediamine reagent
C : fraction C before hydrolysis
C hyd. : fraction C after hydrolysis
C′ hyd. : fraction C after hydrolysis followed by Dowex 1 treatment to remove phosphates
Rib : ribose, Fr : fructose, Ga : galactose,
Man : mannose, Gl : glucose, G6P : glucose-6-phosphate

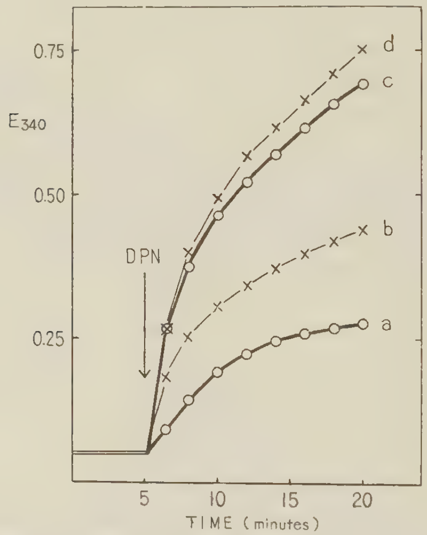
be α-glycerophosphate. Unfortunately, the authors could not obtain a pure sample of α-glycerophosphate, so both β-glycerophosphate and a mixture of α- and β-glycerophosphate were used side by side as the standards. Then, in all analysis tried, the Fraction D showed the identical behaviour with α-glycerophosphate assumed from these standards (Table II). As the Fraction D has a more than three times stronger ability to reduce DPN in the presence of muscle L-α-glycerophosphate dehydrogenase than the assumed synthetic mixture of DL-α- and DL-β-glycerophosphate, it was concluded that the major component of Fraction D was L-α-glycerophosphate.

(Muscle L-α-Glycerophosphate Dehydrogenase)

$\text{L-}\alpha\text{-Glycero-P}+\text{DPN}^+ \rightarrow \text{Dihydroxyacetone-P}+\text{DPNH}+\text{H}^+$

↓

Trapping with Hydrazine



	(c) Fr. D	(d) α, β-mixed GP	(b) β-GP Commercial
DPNH Formed/P	1	less than 0.3	less than 0.1

FIG. 7. Reduction of DPN by fraction D in the presence of L-α-glycerophosphate dehydrogenase and hydrazine.

curve (a) control

(b) commercial β-glycerophosphate (molecular ratio: ca. 3)

(c) fraction D (molecular ratio: 1)

(d) synthetic mixture of α- and β-glycerophosphate (molecular ratio: ca. 3.5)

Some of the results of these analyses are shown in Figs. 5, 6, and 7.

Fraction A, the Dowex 1 formate unadsorbable radioactivity, was found to be adsorbed on Dowex 50 (-H form) in preliminary experiments. The adsorbed radioactivity could be eluted by dilute HCl and resolved into two fractions, namely A1 and A2. Each of these was purified by repeated adsorption on Dowex 50 column and elution with dilute HCl (Scheme 2,

SCHEME 2

Purification of Fraction A

Rat Liver PCA Extract (obtained from 20 g. of the Tissue)
↓
Treatment with Dowex 1 Formate
↓
Unadsorbable Fraction (Crude Fr. A)
↓
1st Adsorption on Dowex 50 (-H Form, freshly prepared, 1 × 25 cm)
↓
Elution (gradient to 0.05 N HCl)

Crude Fr. A1
↓
Lyophilization
↓
2nd Adsorption on Dowex 50 (-H form, 0.6 × 40 cm)
↓
Elution (gradient to 0.05 N HCl)
↓
Lyophilization
↓
Purified Fr. A1
↓
Analysis

Crude Fr. A2
↓
,,
↓
,,
↓
,,
↓
,,
↓
Purified Fr. A2

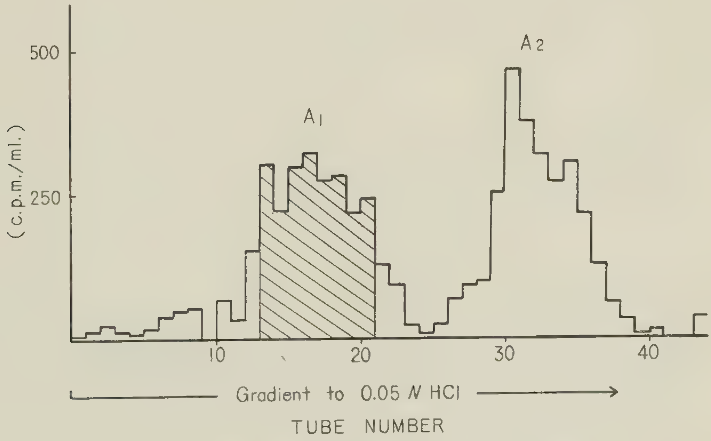


FIG. 8. Column chromatography of the Dowex 1 unadsorbable fraction with Dowex 50.

5 rats were injected with each 60 μc of P^{32}

After 30 minutes, the acid-soluble extract was prepared as described above (total 140 ml.). The extract was passed through a Dowex 1 formate column, and 60 ml. of the unadsorbed fraction was applied to a Dowex 50 column.

column : Dowex 50 (X4, -H form) 1 × 25 cm.,
mixer volume : 150 ml.,
1 tube : 4 ml.

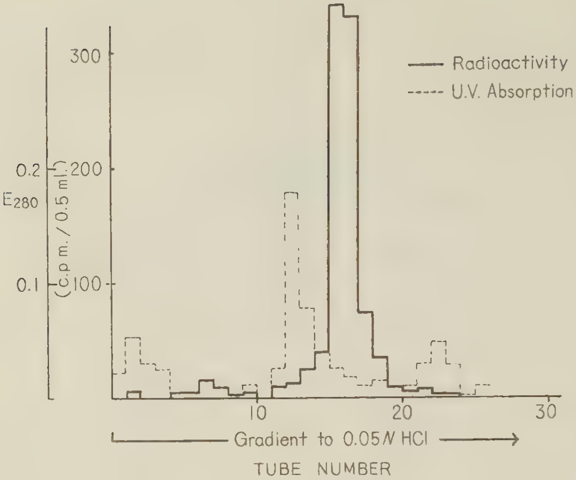


FIG. 9. Rechromatography of fraction A1 with Dowex 50.

Eluates of tube No. 14 to 21 in Fig. 8 were combined, lyophilized, and once more applied to Dowex 50 column.

column :	Dowex 50 (X4, -H form) 0.6×40 cm.
mixer volume :	150 ml.
1 fraction :	4 ml.

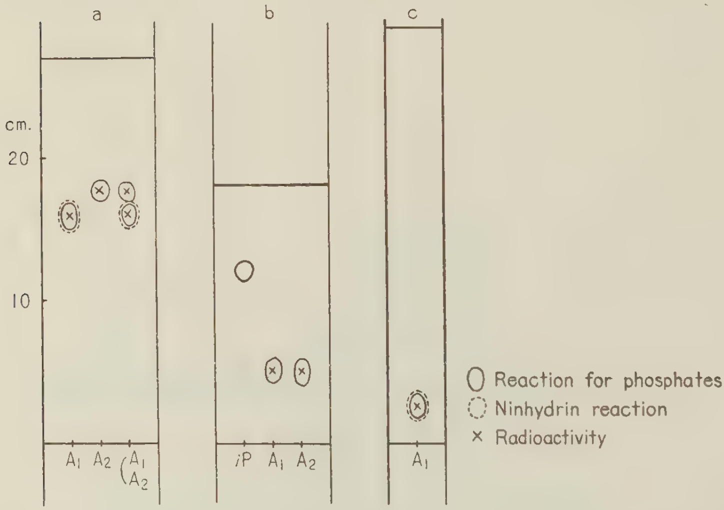


FIG. 10. Paper chromatography of A1 and A2.

(a) Methanol-NH_3-Water (60 : 10 : 30), 14 hours at room temperature.

(b) *tert*-Butanol-Picric Acid-Water (80 : 2 : 20), 20 hours at room temperature, *i*P : inorganic phosphate. (In this solvent the ninhydrin test could not be carried out.)

(c) *n*-Butanol-Acetic Acid-Water (4 : 1 : 1), 19 hours at room temperature.

Figs. 8 and 9). Each was proved to be a single substance by paper chromatography (Fig. 10). A1 was positive with phosphate spot test and ninhydrin spot test. A2 was positive with phosphate spot test but negative with ninhydrin spot test. Either of them showed no U. V. absorption.

Fraction A1 was hydrolysed with 6 *N* HCl at 100° for 48 hours and the hydrolysate was chromatographed with two dimensional system (Fig. 11). By the hydrolysis, it liberated 80 per cent of the bound phosphorus as inorganic phosphate, and also liberated at least three ninhydrin positive substances. The ninhydrin positive substance which was largest in quantity (spot 2 in Fig 11) was examined with two kinds of one dimensional paper chromatography. Furthermore, its dinitrophenyl derivative was prepared and analysed by one and two dimensional paper chromatography. In these chromatographic tests, the major component behaved very similar to glutamic acid, but strict identification was not accomplished yet. The ninhydrin

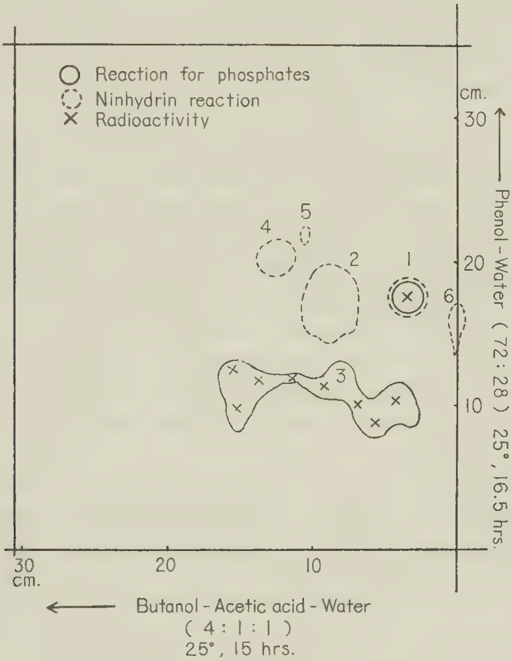


FIG. 11. Two dimensional paper chromatography of purified A1 after hydrolysis.
hydrolysis : in 6 *N* HCl, 97°, for 48 hours liberation
of inorganic phosphate : 83 per cent
1 : A1 remained unhydrolysed,
2 : presumed to be glutamic acid,
3 : inorganic phosphate,
4–6 : ninhydrin positive substances unidentified.

positive spots appeared on hydrolysis were presumed to be amino acids, and the precise nature of fraction A1 is under investigation.

Fraction A2 is not throughly studied yet, so the nature of this substance will also be reported later.

DISCUSSION

Although the semi-micro method presented here has less ability to resolve each nucleotide than the original macro method, it can be sufficiently used for analysing various tissues of individual rats. Even very small organ such as thymus of rat could be analysed by this modified method. Thus it makes possible to compare the distribution pattern of acid-soluble nucleotides in various tissues. Furthermore, by the use of radioactive P^{32}, it is also possible by this method to compare the turnover of various phosphorus compounds in various tissues under normal or pathological conditions. Experiments with other tracers could also be carried out by the use of this method.

Concerning the preparation of the tissue extracts, it is better to use liquid air or liquid nitrogen for freezing sample tissues or, especially, for freezing *in situ* of the whole animal. Because, the *post mortem* change of the phosphorus compounds can be avoided only by rapid freezing. In some tissues the change is rapid and in others slow. The remarkable changes are dephosphorylation of nucleoside-triphosphates and increase of nucleotide-monophosphates (*7*). Unfortunately, the authors usually could not obtain liquid air or nitrogen, but had some chances to utilize liquid nitrogen and to compare the results. The precise comparison will be reported later. At present, it can be said that, at least, for analyses of brain and kidney tne freezing *in situ* is necessary.

As to the preparation of the resin column, many efforts were made to obtain a good resolution of the nucleotides. The 0.6×40 cm. column is relatively longer than the originally described columns. However, it seemed that the longer column yielded better resolution of each nucleotide than the shorter ones under the conditions of the semi-micro method. Glucose-6-phosphate and L-α-glycerophosphate could also be resolved only when the column was prepared freshly, the volume of the perchloric acid extract was not too large, and the length of the column was not too short.

Among the three substances identified here, two of them, glucose-6-phosphate and L-α-glycerophosphate, are already known to exist in liver tissue. But the identification on the chromatogram may contribute to the studies of analysing their actual turnover and metabolism in various tissues under various conditions.

Acid-soluble phosphorus compounds containing amino acid or related compounds were occasionally described in literature. Among these, those which have stable phosphate and no U. V. absorption are O-phosphoserine in rat liver and other tissues (*25*, *26*), serine-ethanolamine O-phosphodiester in turtle tissue (*27*), and ethanolamine O-phosphoric ester in rat tissues and human tumors (*28*, *29*). The compound reported in this paper is presumed to contain amino acids and shows relatively high turnover rate of the phosphate. Further investigation is necessary to elucidate the structure and function of this substance.

SUMMARY

1. A modified semi-micro gradient elution chromatography for the analysis of tissue acid-soluble phosphorus compounds was described. One to 3 g. of tissue could be analysed by this method.

2. Liver, muscle, and thymus tissue of rats were analysed and the chromatograms were illustrated.

3. The distribution and turnover of the acid-soluble phosphorus compounds in rat liver were also studied by P^{32} injection with the use of the semi-micro meteod.

4. Glucose-6-phosphate and L-α-glycerophosphate were identified on the chromatogram, and a phosphorus compound containing amino acid was separated from the Dowex 1 formate unadsorbable fraction.

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MECHANISM OF CYANIDE RESISTANCE IN ACHROMOBACTER

I. ADAPTIVE FORMATION OF CYANIDE RESISTANT RESPIRATORY SYSTEM IN GROWING CELLS

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As it is well known, microorganism have an ability to exhibit a characteristic adaptability to their environment. The drug resistance in microorganisms is one of the most typical phenomenon of this kind. Though in many cases, the drug resistance was reported to be caused by genetic changes, *i.e.*, mutation, many studies have been reported concerning the nongenetic, physiological adaptation for drugs. About many drugs, various types of the resistant mechanisms have been known. However, little has been known concerning why and how such a resistant system is formed.

In order to study how microorganisms adapt to their environment, how they form a resistant system for drugs, the mechanism of cyanide resistance in microorganisms has been studied enzymologically.

In the previous paper (*1*), it was reported, that *Aerobacter cloacae* isolated from soil could grow in the cyanide containing medium adaptively, and formed a cyanide resistant respiratory system adaptively. However, it was unable to make clear the biochemical changes during the adaptation.

This paper reports studies on the adaptive formation of a cyanide resistant respiratory system in growing cells, particularly with regard to the quantitative changes in the terminal respiratory components.

EXPERIMENTALS

Cultivation—Bacteria were aerobically grown in a bouillon medium (pH 7.0), containing meat extract 1 per cent, peptone 1 per cent and NaCl 0.5 per cent in a one to one mixture of distilled water and tap water. The growth rate of bacteria were measured using a nepherometer. The bacteria were harvested at the early stage of the logarithmic growth phase (about $7\sim8\times10^8$ cells/ml.).

Preparation of Cell-Free Extract—Bacterial cells were collected by centrifugation from 300 ml. (2 liters in the case of cytochrome estimation) aerobic culture medium contained $7\sim8\times10^8$ cells per ml. After washing with potassium chloride solution (0.5 per cent), the cells were suspended in 30 ml. of $M/15$-phosphate buffer (pH 7.4) which contained 0.5 per cent potassium chloride. This suspension was treated with sonic oscillator (10 KC) for 15 minutes and centrifuged ($5{,}000\times g$, for 30 minutes) to remove the intact cells. The sonicate thus obtained was centrifuged at 40,000 r.p.m. for 60 minutes in an Spinco ultracentrifuge (Model L, head No. 40). The clear supernatant fluid thus obtained was decanted out and the dark reddish particulate precipitate was suspended in 30 ml. of $M/15$-phosphate buffer (pH 7.4). The supernatant fluid and the particulate fraction were used as sources of the oxidases. These preparations possessed a oxidative activity towards succinate and DPNH. The rate of DPNH oxidation by the cell-free preparation was nearly equal to that of glucose oxidation by the corresponding amount of intact cells. These preparations also possessed a catalase activity which is strongly inhibited by cyanide $10^{-3}\,M$).

In the case of the estimation of cytochromes, the particulate precipitate was suspended in 5 ml. of $M/15$-phosphate buffer and was stood for one or two hours in aerobic conditions.

Measurement of Oxidative Activity—In resting cells, the oxidative activity for several substrates was estimated using Warburg respirometer at 30°. When the subcellular preparation was used, succinoxidase activity was also measured using Warburg respirometer, while DPNH oxidase activity was usually estimated by measuring the change of optical density at 340 mμ using a spectrophotometer.

Estimation of Cyanide in Medium—Cyanic acid in a medium was estimated using the colorimetric method of Snell and Snell (*2*).

Estimation of Cytochromes—Cytochrome components were detected spectrophotometrically based on the opal glass method (*3*), using Cary Model 14 Spectrophotometer. The relative amount of cytochrome b was estimated from the difference in optical density between 561 mμ and 545 mμ, in the curve of difference spectrum (reduced minus oxidized) while the relative amount of cytochrome a_2 was estimated from the difference in optical density between 630 mμ and 650 mμ in the curve of difference spectrum (reduced minus oxidized).

The comparison of the amount of cytochromes with cyanide resistant and non-resistant cells was carried out using the same amount of cells in weight.

Detection of Hydrogen Peroxide—As hydrogen peroxide liberates iodine from potassium iodide in acetic acid solution, hydrogen peroxide was qualitatively detected by iodin-starch reaction.

Determination of Flavin Compound—After acid hydrolysis of the sample with 0.1 N HCl at 115° for 20 minutes, the total flavin content was assayed by means of microbial method (*4*).

Materials—DPN was a sample from Nutritional Biochemicals Corporation. Alcohol dehydrogenase was prepared from yeast by the method of Racker (*5*). DPNH was prepared by reducing DPN with alcohol dehydrogenase (*6*). Purified D-amino acid oxidase preparation (catalase free) was kindly supplied from Mr. Ozawa, Faculty of Medicine, University of Nagoya.

RESULTS

Isolation and Identification of Cyanide Resistant Bacteria—Three strains of bacteria which could grow in the bouillon medium containing $10^{-3}\,M$ of potassium cyanide, were newly isolated from soil after the previous work (*1*). One of them which could grow in the cyanide containing medium rather rapidly, was found to belong to *Achromobacter* according to Bergey's Mannual of Determinative Bacteriology (*7*). The following experiments were carried out using *Achromobacter* strain 7.

Effect of Cyanide on Bacterial Growth—Fig. 1 shows the growth of *Achromobacter* in the bouillon medium in the presence or absence of 10^{-3} *M* of cyanide, respectively. In the presence of cyanide, though the lag period was remarkably prolonged, significant growth inhibition was not observed at the logarithmic growth phase. At the logarithmic growth phase, almost all amount of cyanide still remained in the medium. These results show that the bacteria have an ability to exhibit cyanide resistant growth adaptively.

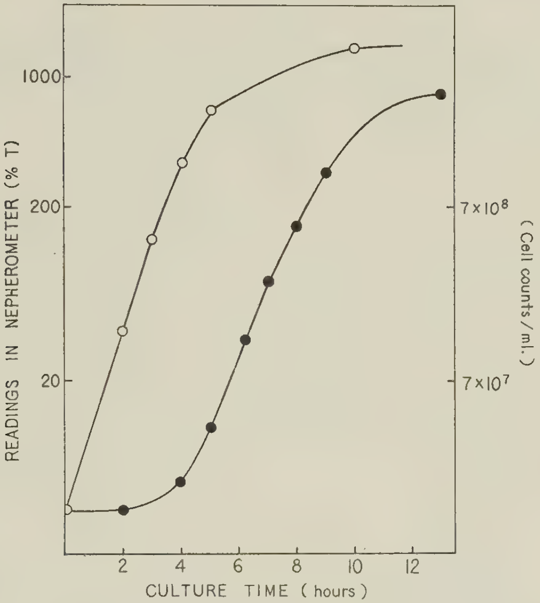

FIG. 1. Effect of cyanide on growth in bouillon medium. Cultivation was carried out on Monod's cultivation apparatus. —○— without cyanide, —●— with 10^{-3} *M* of cyanide.

Effect of Cyanide on Respiration of Resting Cells—Effect of cyanide on the oxidation of glucose, succinate and fumarate were measured using intact cells grown in the presence or absence of cyanide. The result is shown in Table I. The respiration of the cyanide non-adapted cells was remarkably inhibited by cyanide, whereas that of the cyanide adapted cells was only slightly inhibited by cyanide, indicating the adaptive formation of a cyanide resistant respiratory system. In addition, Q_{O_2} of the cyanide adapted cells were about four third times as large as that of the cyanide non-adapted cells. In the following part of this report, the word 'adapted cells' means the cells grown in the cyanine containing medium, and the word 'non-adapted cells' means the cells grown in the cyanide free medium.

Disappearance of Cyanide Insensitivity in Adapted Cells—As shown in Fig. 2 so long as the adapted bacteria were cultured in the cyanide containing medium, the cyanide insensitivity in their respiration was maintained a

TABLE I

Effect of Cyanide on the Respiration of Resting Cells

Substrate	Inhibition by 10^{-3} M of cyanide	
	Adapted cells	Non-adapted cells
Glucose	25%	95%
Succinate	20	100
Fumarate	30	100

Each Warburg vessel contains, 4×10^9 cells, 0.2 ml. of 10 mM KCN and 1.6 ml. of $M/15$ phosphate buffer (pH 7.4) in main compartment, 0.2 ml. of 0.2 M of substrate in side arm and 0.2 ml. of 20 per cent KOH in center well. Total volume 2.2 ml. Reaction temperature 30°.

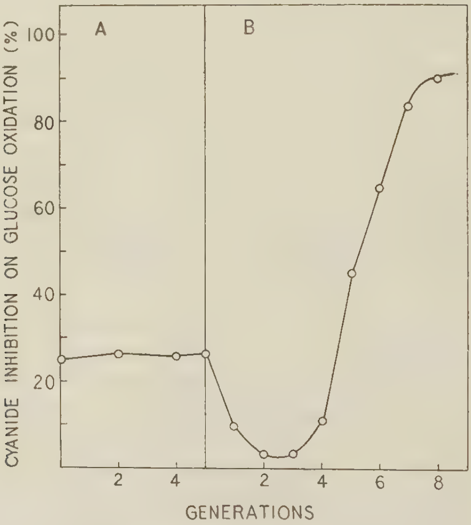

FIG. 2. Disappearance of cyanide insensitivity.
A; In cyanide (10^{-3} M) containing bouillon medium.
B; In cyanide free bouillon medium.
Conditions were the same given in Table I.

constant level (About 25~30 per cent inhibition). When the adapted cells were transplanted and grown in the cyanide free medium, the cyanide insensitivity in their respiration was varied as follows. Contrary to expectation, the insensitivity to cyanide has rather increased during the first to the third generation, and suddenly decreased during the fifth to the seventh generation. Then after the eight times division, cells showed typical cyanide sensitive respiration.

Localization of Succinate and DPNH Oxidase Systems in Cells—It is of importance to determine what part of the respiratory system are altered during the adaptive formation of the cyanide resistant respiratory system.

Therefore, further studies were carried out using cell-free extracts as an enzyme preparation. The oxidase activity of the supernatant fluid and the particulate fractions towards DPNH and succinate was shown in Table II.

TABLE II

Localization of Succinate and DPNH Oxidase Systems in Cells

		Supernatant fluid (S)	Particulate fraction (P)	(S)+(P)
Adapted cell	Succinoxidase activity[1]	0.016	0.35	0.23
	DPNH oxidase activity[2]	0.026	0.116	0.173
Non-adapted cell	Succinoxidase activity[1]	0.02	0.35	0.27
	DPNH oxidase activity[2]	0.015	0.190	0.280

1) Oxygen uptake μAtom/mg. Nitrogen/min.

2) $-\Delta OD_{340\ m\mu}$/mg. Nitrogen/min.

Succinoxidase activity was measured using Warburg respirometer at 30°. Each Warburg vessel contained 0.5 ml. of supernatant fluid, 0.5 ml. of particulate fraction or sum of these two as an enzyme sources and 1.3 ml. (or 0.8 ml.) of $M/15$-phosphate buffer (pH 7.4) in main compartment, 0.2 ml. of 0.2 M-succinate solution in side arm and 0.2 ml. of 20% KOH in center well. Total volume 2.2 ml.

DPNH oxidase activity was measured spectrophotometrically at 20°. Each cuvette contained 0.2 ml. of supernatant fluid, 0.2 ml. of particulate fraction or sum of these two as an enzyme source and 2.3 ml. (or 1.8 ml.) of $M/15$-phosphate buffer (pH 7.4). The reactions were started by adding 0.2 ml. of 2 mM-DPNH solution in the cuvette.

Though the large part of the oxidase activity were located in the particulate fraction in both cases, a detectable activity was also observed in the supernatant fluid. In the case of succinate oxidation, the supernatant fluid inhibited the oxidase activity of the particulate fraction, whereas in the case of DPNH oxidation, addition of the supernatant fluid showed a stimulative effect on the oxidase activity. This fact indicates that the supernatant fluid contains some essential component of the DPNH oxidizing system. However, any possible component such as cytochrome has never been detected in the supernatant fluid.

Effect of Cyanide on Oxidation of DPNH and Succinate—As shown in Table III, the preparations obtained from the non-adapted cells were strongly inhibited by cyanide, while the preparations obtained from the adapted cells were weakly inhibited by cyanide.

Table IV shows the relation between the concentration of cyanide and the degree of inhibition of DPNH oxidase system. The preparation obtained from the non-adapted cells were more strongly inhibited by cyanide than that obtained from the adapted cells. However, it is noticeable that even in the case of non-adapted cells, its sensitibity towards cyanide was not so high than that of mammalian oxidase system.

Absence of Hydrogen Peroxide as a Produced of Oxidative Reaction—As a cyanide

TABLE III

Effect of Cyanide on the Oxidation of DPNH and Succinate by Cell-free Extracts

	Enzyme preparation[1]		Inhibition (KCN 10^{-3} *M*)
DPNH oxidase activity	Non-adapted cell	S	79%
		P	90
		S+P	93
	Adapted cell	S	33
		P	15
		S+P	28
Succinoxidase activity	Non-adapted cell	P	72
	Adapted cell	P	20

1) S: Supernatant fluid P: Particulate fraction

Conditions were similar to those given in Table II.

TABLE IV

Effect of Cyanide Concentration on DPNH Oxidation by Crude Extracts

Cyanide concentration	Inhibition of DPNH oxidation	
	Non-adapted cell	Adapted cell
10^{-2} *M*	98%	81%
5×10^{-3}	97	57
10^{-3}	88	41
5×10^{-4}	82	28
10^{-4}	65	21
5×10^{-5}	57	—

Condition were similar to those given in Table II.

insensitive terminal oxidase, the flavin enzyme such as DPNH oxidase (*8*) or D-amino acid oxidase has been known. In the case of flavinnucleotide-linked oxidase, the production of hydrogen peroxide during the oxidative reaction is characteristic. Therefore, using a well-washed particulate preparation (washed two times in Spinco L) of adapted cells, the detection of hydrogen peroxide during the terminal oxidation was tried in the presence of 10^{-3} *M* of cyanide. Besides the washed particulate preparation, D-amino acid oxidase was added to the enzyme preparation to make both activities nearly equal with regard to the rate of oxygen uptake. Table V shows the production of hydrogen peroxide during the oxidative reaction in the presence of DPNH, succinate or D-alanine as a substrate. Using DPNH or succinate as a substrate, production of hydrogen peroxide (less than 0.02×10^{-6} M) was not detected, whereas in the case of D-alanine, a remarkable production of hydrogen peroxide was observed. This result strongly suggests that DPNH and succinate are oxidized by oxygen through one-electron carrier (probably

TABLE V

Non-production of Hydrogen Peroxide during the Terminal Oxidative Reaction

Reaction mixture	Substrate	O_2 uptake	H_2O_2 formed
		μAtom/10 min.	
Washed particulate preparation (0.2 ml.)	Non	0	−(0.02 μM)
D-Amino acid oxidase (0.2 ml.)	succinate	3.5	−(,,)
10 mM KCN (0.2 ml.)	DPNH	6.3	−(,,)
50 mM Substrate (10 μM) (0.2 ml.)	D-Alanine	4.9	+
Phosphate buffer (M/15, pH 7.4) (1.4 ml.)	H_2O_2 (1 μM)	0	+
Total Volum 2.0 ml.			

Reactions were carried out in Warburg vessel at 30°. Each Warburg vessel contained 0.2 ml. of 20% KOH in center well.

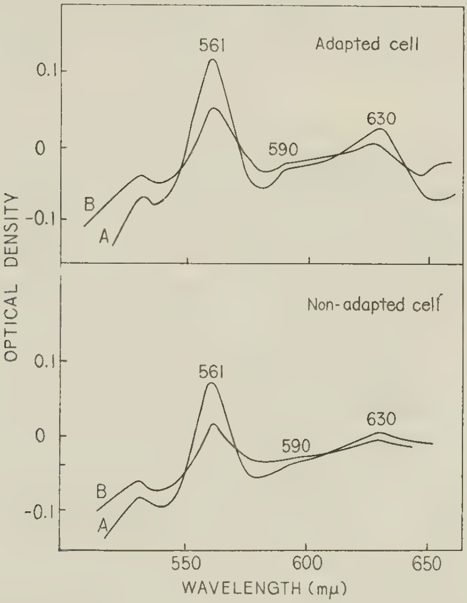

FIG. 3. Difference spectra of particulate fraction (reduced minus oxidized).

A ; Reduced with dithionite, B ; Reduced with succinate.

cytochrome system) in this bacteria.

Spectrophotometric Analysis of Cytochrome System—Fig. 3 shows the differences spectra (reduced-oxidized) of the particulate fraction prepared from both the adapted and the non-adapted cells. In both case, the absorption maxima corresponding to the α-band of cytochrome b (561 mμ), a_1 (590 mμ) and

a_2 (630 mμ) were observed. The absorption peak around 530 mμ may be corresponded to the β-band of cytochrome b. However, it is noticeable that the amount of cytochrome a_2 in the adapted cell preparation was about ten times as large as that of non-adapted cell preparation. Though the amount of cytochrome a_1 in the preparation was very small comparing with that of cytochrome a_2, its amount was also remarkably increased after the adaptation. As to the soluble fraction, though the similar spectra of cytochromes were observed, their amounts were very small comparing with that of the particulate preparation.

Nature of Other Components of Electron Transport System—Using methylene blue as a hydrogen acceptor in anaerobic state, the cell-free preparation dehydrogenated DPNH and succinate through the mediation of flavoprotein. The rate of dehydrogenation of succinate is fairly slower than that of DPNH. These dehydrogenase reactions were found to be cyanide insensitive in both preparations obtained from the adapted and the non-adapted cells. In addition, there is no remarkable difference in flavin content between the adapted cells and the non-adapted cells. These results strongly suggest that the flavin component of electron transport system is insignificant with regard to the mechanism of the cyanide resistance.

DISCUSSION

First of all, it is important to decide whether this phenomenon is a physiological adaptation or a genetic mutation. From following findings, this phenomenon is thought to be a kind of physiological adaptation: a) The lag period in Fig. 1 caused by the addition of cyanide is only 3 hours. If this phenomenon is due to a kind of mutation, we are forced to consider that more than ten per cent of the inoculated cells are mutated as soon as they are contact with cyanide. However, it could not be considered that $10^{-3}\,M$ of cyanide possesses such a mutagenic activity. b) When the cells were suspended in the thin plate of bouillon agar, the number of colonies appeared after two days were almost the same in both the cyanide containing and the cyanide free medium, though the rate of the development of colonies was fairly slow in the case of cyanide containing medium. This shows that the all cells became resistant to cyanide in the medium. c) As will be reported in the following papers (*9*), the respiration of cells could become cyanide insensitive without growth.

Second problem is a nature of the cyanide insensitivity in electron transport system, namely how the terminal oxidase system became cyanide insensitive adaptively. The electron transport system of this bacteria contained flavoprotein, cytochrome b, a_1 and a_2 but did not contained cytochrome c, a and a_3. This cytochrome composition is similar to that of *Escherichia coli* or *Aerobacter aerogenes* and cytochrome a_2 was thought to be a terminal oxidase in these bacteria.

Cyanide may inhibit the action of cytochrome a_2 or a_1. This respiratory chain is also exist in the adapted cells, and no qualitative difference in respiratory chain could be observed between the adapted and the non-adapted cells. As shown in Table III and IV, in the presence of 10^{-3} *M* of cyanide, the electron transport system of the non-adapted cells acts weakly but detectably (about 10 per cent of the activity of the case of cyanide free in DPNH oxidation). As cyanide may inhibit the action of cytochrome a_2 or a_1, these cytochrome have a possibility to act in the presence of 10^{-3} *M* of cyanide, though the activity is very weak. Therefore, the more the amount of cytochrome a_2 or a_1 increases, the stronger its activity in the presence of cyanide becomes. On the other hand, using DPNH or succinate as a substrate, there were no remarkable increase in Q_{O_2} during the adaptation to cyanide as shown in Table II. This fact indicates that there is some components which limit the action of respiratory chain other than cytochrome a_1 or a_2, such as flavoprotein or cytochrome b. The increase in enzymatic activity of cytochrome a_2 or a_1 in the presence of cyanide without remarkable increase in total oxidase activity of electron transport chain, means the increase of cyanide insensitivity of the whole respiratory system, though cytochrome a_2 and a_1 are still strongly inhibited by cyanide. This may be a mechanism of cyanide resistance in this bacteria. The figurative explanation of this mechanism is as follows.

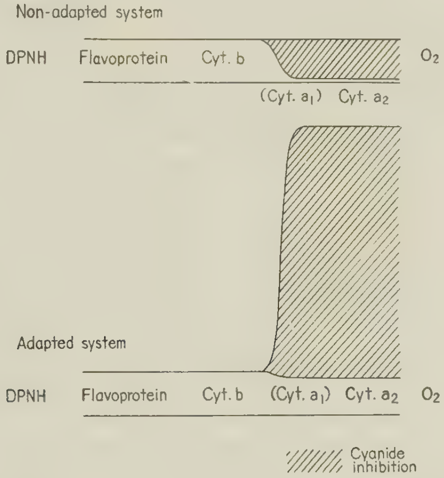

FIG. 4. Mechanism of cyanide resistance. Cyanide inhibition.

So far, many mechanisms have been reported concerning the drug resistance in bacteria including the case of genetic mutation; formation of a drug-insensitive enzyme, formation of drug-destroying enzyme, formation of a drug-inactivating substance and increasing formation of an antagonist of the drug (in the case that the drug is an antimetabolite). The mechanism found in this study does not belong to any of these mechanisms. Here, a new type of mechanism in drug resistance was presented, namely an increasing formation of an enzyme which is partially inhibited by the drugs.

SUMMARY

1. One strain of *Achromobacter* isolated from soil was found to show a cyanide resistant growth adaptively in the presence of 10^{-3} M of cyanide.

2. The respiration of the cyanide adapted cells was cyanide insensitive, whereas that of the cyanide non-adapted cells was remarkably inhibited by cyanide.

3. The oxidation of DPNH and succinate by the cell-free preparations obtained from the cyanide non-adapted cells was remarkably inhibited by cyanide, whereas that obtained from the adapted cells was less inhibited.

4. The DPNH or succinate oxidase activity was almost located in the subcellular particulate fraction, and the terminal oxidase was found to be a one electron carrier.

5. Cytochromes b, a_1, and a_2 were found in the particulate fraction in both the cyanide adapted and the non-adapted cells. However, the amounts of cytochrome a_2 (and a_1) were greatly increased during the adaptation to cyanide.

6. It was postulated that the mechanism of cyanide resistance in the bacteria was caused by the increase of cytochrome a_2 (or a_1) which may be strongly inhibited by cyanide in nature. This is a new type of the mechanism in the drug resistance.

The authors wish to express their sincere appreciation to Dr. K. Sakaguchi for his kind guidance throughout this work. Thanks are also due to Mr. K. Komagata for his kind advice in the diagnosis of bacteria, to Mr. S. Miyazawa for the assay of riboflavine, and to Miss. M. Nakano for the isolation of the bacteria used.

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ACTIONS OF RADIATIONS ON ENZYMES

III. THE INACTIVATING MECHANISM OF SULFHYDRYL ENZYMES BY γ-IRRADIATION

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(Received for publication, September 8, 1959)

Sulfhydryl enzymes have already been reported to be susceptible to ionizing radiations and to become inactive by irradiation of a small dose (*1*, *2*). In the previous papers (*3*, *4*) inactivation of urease, yeast alcohol dehydrogenase (ADH) and liver glutamic dehydrogenase (GDH) was reported to be caused by γ-rays when they were irradiated in aqueous solutions. Inhibition of the enzymes caused by the relatively moderate γ-irradiation was found to be reactivated reversibly by the addition of thiol compounds such as cysteine and reduced glutathione, while inactivation caused by larger doses of γ-rays was proved to be irreversible (*3*, *4*). Reversible inactivation of sulfhydryl enzymes such as phosphoglyceraldehyde dehydrogenase, adenosinetriphosphatase and yeast hexokinase by irradiation of X-rays was studied by Barron and his collaborators (*1*, *2*) and elucidated to be attributed to reversible oxidation-reduction between active sulfhydryl groups and corresponding disulfides in the enzyme molecules. Measurable decrease in amounts of the sulfhydryl groups in enzyme protein in the oxidation of the enzymes by γ-irradiation should be expected.

It has been well established that when water is irradiated with ionizing radiations there occurs a decomposition of molecule of water to hydrogen atom (H) and hydroxyl radical (OH) (*5*, *6*). When a sizable amount of oxygen is dissolved in water, formation of hydrogen peroxide and hydroperoxyl (O_2H) radical is demonstrated to occur (*7*). Owing to these powerful oxidizing entities radiolysis of radio-sensitive substances is especially stimulated in their aqueous solutions. Inactivation of enzymes in aqueous solutions by γ-irradiation, therefore, should be ascribed to a similar action of the radicals produced from water (*8*, *9*).

The present paper is concerned with the actions of reacting entities on the enzymes and the correlation between decrease of amounts of sulfhydryl groups and degree of the inactivation of enzymes.

EXPERIMENTALS

Enzyme Preparation—As the representatives of sulfhydry enzymes, urease, yeast alcohol dehydrogenase and liver glutamic dehydrogenase together with catalase as a non-sulfhydryl enzyme were chosen for the present investigation. Preparation of these enzymes was carried out as follows: crystalline yeast alcohol dehydrogenase (ADH) was obtained from fresh baker's yeast (Oriental Yeast Co. Ltd., Osaka) by the method of Racker (*10*) with modification*. The specific activity of the ADH preparation was 5×10^4 units per mg. of protein. Crystalline urease was prepared from Jack bean meal of reddish species harvested in Japan by the methods devised by Sumner (*11*), Hellerman (*12*), and Dounce (*13*). The specific activity of recrystallized urease was 1.0×10^5 Sumner units per g. of protein. Liver glutamic dehydrogenase (GDH) was obtained from fresh beef liver by the methods of Olson (*14*), Strecker (*15*), and Iwatsubo *et al.* (*16*). The activity of the GDH preparation was 1.1×10^3 units per mg. of protein. Crystalline catalase was prepared from fresh beef liver by Shirakawa's method (*17*). The Kat. f. of the catalase preparation was 5.0×10^3.

Gamma Irradiation—The γ-ray source used in this work was Co^{60} (50c.) in a Toshiba RIT-1 Teletherapy Unit. Dosage of γ-irradiation was determined by Fricke's ferrous-ferric chemical dosimetry (*18*). The dose rate was 800r. per minute. The γ-irradiation technique applied in this experiments was the same as that described in the previous papers (*3*, *4*).

Determination of Sulfhydryl Groups in Enzyme Molecules—Amounts of sulfhydryl groups in enzyme proteins were determined colorimetrically by using a *p*-chloromercuribenzoate reagent according to Boyer (*19*). A new method for determination of sulfhydryl groups which was devised by Ozawa and Egashira (*20*) was also applied**. The procedure of this method is as follows: An adequate amount of Ruheman's purple*** is dissolved in water to show an absorbance of 4.0 or 5.0 at 570 mμ by a Beckman DU spectrophotometer. To 45 ml. of the solutions, 5 ml. of a 1×10^{-3} M mercuric chloride solution is added. In a small separating funnel (10 ml.), 3 ml. of the mixture is shaken with 3 ml. of isopropyl ether. The resulting mixture is allowed to stand with occasional vigorous shaking until decoloration of the aqueous layer. During these treatments mercury complex of Ruheman's purple is formed and deposited out between the two layers. The complex is washed with water completely. After discarding the aqueous layer, 4 ml. of the enzyme solution, of which the content of sulfhydryl groups ranges from 5×10^{-6} to 5×10^{-5} M, is added to the solution, and the mixture is shaken again. By the reaction between sulfhydryl groups and the mercury complex, Ruheman's purple is liberated corresponding in amount to the sulfhydryl groups and the coloration passes over into the water layer. The concentration of sulfhydryl groups can be determined from the amount of Ruheman's purple thus formed.

RESULTS AND DISCUSSION

Action of Irradiation Products of Water on Enzymes—It has been established that when oxygen-containing water is irradiated with ionizing radiations, hydroxyl radical, hydroperoxyl radical and hydrogen peroxide are produced (*21*, *22*). Hydroxyl radical is known to be formed by the reaction of hydrogen atom and dissolved oxygen molecule and hydrogen peroxide is proved to be the recombination product of hydroxyl radicals. As these radicals and hydrogen peroxide are all powerful oxidizing agents, the inactivation of enzymes by radiation caused in aqueous solutions may be attributable to the action of these irradiation products of water on enzymes. As catalase is observed to be resistant to ionizing radiations, the effect of hydrogen peroxide on sulfhydryl enzymes may be eliminated when catalase is present in the medium.

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* In the course of autolysis of yeast cells, toluene was used (*4*); Iwatsubo, personal communications.

** By the courtesy of Mr. Ozawa and Mr. Egashira, the procedure of their method was allowed to be described before publication. The authors wish to express their sincere gratitude for their goodwill.

*** Ruheman's purple is prepared according to the method described by Moore and Stein (*25*). The procedure of recrystallization was modified as follows: Ruheman's purple obtained was washed with an acetate buffer solution (pH 5.0), and dissolved in a large amount of hot water. To this solution, a concentrated sodium acetate solution was added till precipitation of the pigment began to appear. This treatment was repeated three times.

TABLE I

Effect of Hydrogen Peroxide on Inactivation of Yeast ADH by γ-Rays

Reaction system	Activity		Inhibition (%)
	Before treatment (*unit*)	After treatment (*unit*)	
ADH1) + γ-ray, 3×10^4 r.	140	81	42.2
ADH1) + boiled catalase + γ-ray, 3×10^4 r.	140	85	39.3
ADH1) + catalase + γ-ray, 3×10^4 r.	140	101	27.8
ADH2) + H_2O_2, 2.5×10^{-5} *M*	115	105	8.7
ADH3) + catalase + H_2O_2, 2.5×10^{-5} *M*	102	100	1.9

ADH: 1) 0.14 mg.; 2) 0.12 mg.; 3) 0.10 mg.; of yeast ADH was dissolved in 1 ml. of water respectively.

Catalase: this preparation was added to a yeast ADH solution before the treatment with γ-ray or hydrogen peroxide. Boiled catalase was made from the same preparation for estimation of the protecting effect of enzyme protein.

It was observed that the inactivation of yeast alcohol dehydrogenase was protected by catalase* when alcohol dehydrogenase was irradiated by γ-rays. The results are shown in Table I.

Since hydroperoxyl radicals and hydrogen peroxide are formed when molecular oxygen is dissolved in water, the action of γ-rays on enzyme solutions may differ markedly according to the content of molecular oxygen in the solutions (*21*, *22*).

Inhibitory effects of these radicals on yeast alcohol dehydrogenase were examined under the condition in which dissolved oxygen was expelled by bubbling nitrogen gas through the enzyme solutions. The action of OH radical in the solution was also estimated using Fenton's reagent. The results obtained are shown in Table II.

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* Irradiation of the catalase preparation with γ-rays of the doses below 5 kr. was proved to cause no inhibition of the enzyme.

TABLE II

Effect of OH and O_2H Radicals on Inactivation of Yeast ADH by γ-Rays

Reaction system	Activity		Inhibition (%)
	Before treatment (*unit*)	After treatment (*unit*)	
ADH[1] + γ-ray, 3×10^4 r.	103	45	56.3
ADH[1] + N_2	103	89	13.6
ADH[1] + N_2 + γ-ray, 3×10^4 r.	103	62	39.8
ADH[2] + $FeSO_4$, 2.5×10^{-7} *M*	115	113	1.7
ADH[2] + H_2O_2, 2.5×10^{-5} *M*	115	105	8.7
ADH[2] + $FeSO_4$, 2.5×10^{-7} *M* + H_2O_2, 2.5×10^{5} *M*	115	0	100.0

ADH: 1) 0.10 mg. and 2) 0.12 mg. of yeast ADH was dissolved in 1 ml. of water. Nitrogen gas was passed into an enzyme solution for 20 minutes.

From the results presented in Tables I and II, it becomes evident that 24 per cent of the total inactivation of yeast ADH by γ-rays is ascribed to the oxidation of the enzyme by hydrogen peroxide, while O_2H radical participates in 29 per cent of inactivation. Consequently the inactivation due to OH radical is 47 per cent.

Inactivation of Enzymes by Oxidation of Sulfhydryl Groups—As the preliminary experiments, decrease in amounts of sulfhydryl groups of cysteine molecules by γ-irradiation was determined. The results are shown in Fig. 1 and 2.

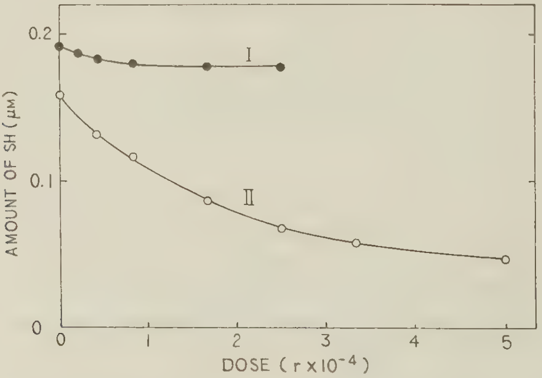

FIG. 1. Decrease in amount of sulfhydryl group of cysteine caused by γ-irradiation (I).

Curve I shows the result obtained at pH 4.2 and curve II that at pH 8.0; Cysteine concentration was 2×10^{-3} *M*; Determination of sulfhydryl groups was carried out by Boyer's method.

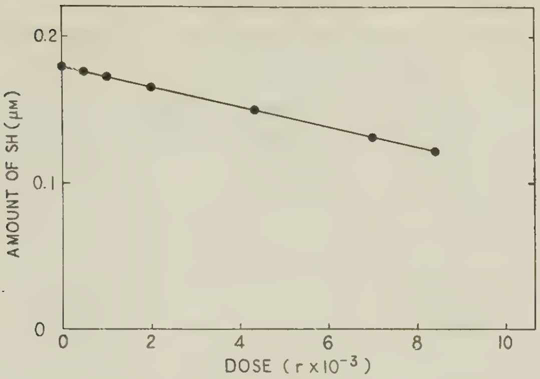

FIG. 2. Decrease in amount of sulfhydryl group of cysteine caused by γ-irradiation (II).

1×10^{-3} *M* solution of cysteine of pH 7.0, was used: Determination of sulfhydryl groups was carried out by Boyer's method.

A linear correlation was observed to hold between the decrease of thiol groups and the dose of γ-rays within 8 kr. of dose.

Several enzymes in which sulfhydryl groups have been demonstrated to be active groups were found to be easily inactivated even by moderate doses of ionizing radiations (*1–4*). It has been agreed that this inactivation is attributable to the susceptibility of sulfhydryl groups to oxidation into disulfides by the oxidizing agents produced by irradiation (*1*, *2*). Barron proposed the following scheme as a mechanism for the inactivation of sulfhydryl enzymes by X-rays in aqueous solution (*1*, *2*).

$$2ESH+2OH=ES-SE+2H_2O$$
$$2ESH+2O_2H=ES-SE+2H_2O_2$$
$$2ESH+H_2O_2=ES-SE+2H_2O,$$

where ESH and ES-SE stand for reduced (active) and oxidized (inactive) enzyme, respectively. According to this view measurable changes in the amount of sulfhydryl groups should be expected with the progress of the inactivation of enzymes.

Actually decrease occurred in the amount of sulfhydryl groups of crystalline urease, crystalline yeast alcohol dehydrogenase, and liver glutamic dehydrogenase respectively during the irradiation of γ-rays as determined colorimetrically. Results are given in Table III. The relationship between the depression of urease activity and the decrease of sulfhydryl groups of the enzyme is shown in Fig. 3.

It has been reported in the previous paper (*3*) that the inactivation of urease by γ-irradiation could be recovered by the addition of thiol compounds such as cysteine and reduced glutathione. The rate of recovery in activity of which had been urease preparations inactivated by different doses of γ-rays was investigated. The results are presented in Table IV.

TABLE III

Change in Amount of Sulfhydryl Groups of Enzyme Proteins Caused by γ-Irradiation

Enzyme	Dose (*r*)	Amount of SH groups		Decrease (%)
		Before irradiation (*M*)	After irradiation (*M*)	
Yeast ADH1), a	3.0×10^{4}	5.38×10^{-8}	4.81×10^{-8}	10.6
,, ADH1), b	,,	1.71×10^{-5}	1.01×10^{-5}	41.3
Liver GDH2)	5.0×10^{2}	1.52×10^{-7}	1.34×10^{-7}	11.7
,,	6.0×10^{3}	,,	1.29×10^{-7}	14.9
,,	1.5×10^{4}	,,	1.23×10^{-7}	19.1
Urease3)	1.3×10^{3}	2.10×10^{-5}	2.05×10^{-5}	2.5
,,	5.0×10^{3}	,,	1.60×10^{-5}	23.8
,,	1.8×10^{4}	,,	1.28×10^{-5}	39.1
,,	3.0×10^{4}	,,	1.18×10^{-5}	43.8

1) 14 μg/0.1 ml.; 2) 0.18 mg.; 3) 6.0 mg. enzyme, was dissolved in 1 ml. of water respectively.

The amount of sulfhydryl groups of ADH^{a} and GDH was determined by Boyer's method, and that of ADH^{b} and urease was done by Ozawa-Egashira's (Ruheman's purple) method.

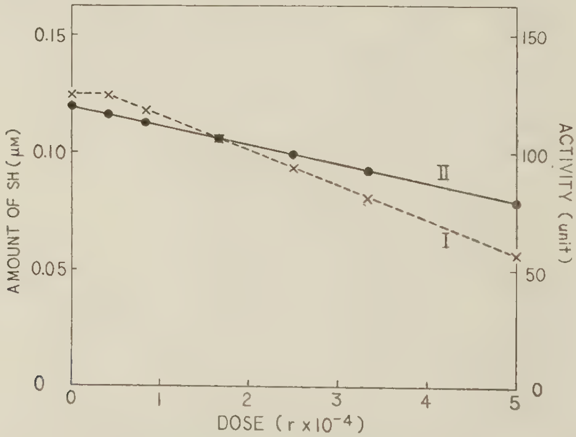

FIG. 3. Inactivation of urease (I) and decrease in amount of sulfhydryl groups (II) caused by γ-irradiation.

1.25 mg. of urease with 125 Sumner units was dissolved in 0.5 ml. of water. The amount of sulfhydryl groups of urease was determined by Boyer's method.

When the doses of the γ-rays applied was below 12 kr., the inactivation of urease caused by γ-irradiation was reversible, while above 15 kr. irreversible inactivation took place more strongly with the increase of doses.

As a result of these experiments not all of the sulfhydryl groups in

TABLE IV

Recoverable and Unrecoverable Inactivation of Sulfhydryl Groups of Urease Caused by γ-Irradiation

Dose (r)	Inactivation (%)	Reactivation (%)	SH Decrease (%)	Recoverable inactivation (%)	Unrecoverable inactivation (%)
8,330	3.0	100.0	1.28	3.00	0.00
12,500	4.5	100.0	1.28	4.50	0.00
16,650	17.3	65.0	3.80	11.2	6.10
25,000	28.5	44.3	17.05	12.6	15.9
33,300	41.0	13.7	30.3	5.62	35.38
37,500	46.8	6.00	25.0	2.81	43.99
41,700	50.0	6.00	30.7	3.00	47.0
45,900	54.5	1.85	43.5	1.00	53.49
50,000	60.0	0.00	56.5	0.00	60.0

1.0 mg. of crystalline urease was dissolved in 0.5 ml. of buffer solution. Determination of sulfhydryl groups was carried out by Boyer's method.

urease molecule were shown to be essential for the activity of the enzyme. Sulfhydryl groups in urease molecule may be classified into two categories: the one is essential and the other non-essential for its activity. Between the amount of decrease in sulfhydryl groups of urease and the degree of inactivation of the enzyme by doses of γ-rays below about 20 kr., a definite correlation was noticed.

SUMMARY

1. Among the oxidizing agents produced by irradiation of γ-rays on water, OH and O_2H radicals and hydrogen peroxide were proved to be effective to cause inactivation of the enzymes whose sulfhydryl groups are responsible for their activity. In the case of yeast ADH, it was shown that 46.5 per cent of the inactivation was caused by OH radical, 29.3 per cent by O_2H radical and 24.2 per cent by hydrogen peroxide.

2. When cysteine in aqueous solution was irradiated by γ-rays the amount of sulfhydryl groups of cysteine molecule was found to decrease linearly with the dose of γ-rays.

3. It was demonstrated that the amounts of sulfhydryl groups in yeast ADH and liver GDH decreased with the increase of the doses of γ-irradiation. In the case of urease, the γ-irradiation caused a lowering of enzyme activity in proportion to the decrease in amount of the sulfhydryl groups.

4. The mechanism of inactivation of sulfhydryl enzymes caused by ionizing radiation as proposed by Barron was confirmed in the case of the inactivation of urease by relatively moderate doses of γ-rays.

The authors wish to express their thanks to Prof. T. Fukuda, Kyoto University, for his kind permission to use the Teletherapy Unit at the University Hospital of Kyoto.

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The Journal of Biochemistry, Vol. 47, No. 3, 1960

TURNIP PEROXIDASE

I. PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF MULTIPLE COMPONENTS IN TURNIP PEROXIDASE*

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(Received for publication, September 14, 1959)

Since Willstätter and his co-workers (*1*) reported about forty years ago that roots of horseradish and turnip are rich sources of peroxidase, the peroxidase of horseradish roots has been extensively studied by many workers (*2, 3*), but little information on the enzyme of turnip roots has been presented. Recently, purification of the enzyme was made by Yamazaki (*4*), who finally succeeded in obtaining it in a crystalline form. However, somewhat earlier than this investigation, Jermyn and Thomas (*5*) reported briefly on the existence of two components of the peroxidase in the juice of turnip roots by means of filter-paper electrophoresis.

The present author has also found by a similar method that at least three kinds of peroxidases are present in the crude extracts of turnip roots, and was able to obtain each component separately in a highly purified state by using zone electrophoresis. This paper describes chiefly the preparative procedures and physicochemical properties of these preparations. Their enzymatic properties will be reported in detail in the succeeding papers.

EXPERIMENTAL

Materials and Methods

Roots of Turnip—The variety of turnip (*Brassica Rapa*) used in this experiment was the one named "Kanamachi-kokabu". Seeds of the turnip were sowed in October and roots were harvested in the latter half of February of the following year at the farm at Mizumoto-koaimachi, Katsushika-ku, Tokyo, from which supplies of turnip for experiment were always obtained. The roots were white, of a slightly flattened spherical shape and of size from 4 to 8 cm. in diameter.

Filter-paper Electrophoresis—Filter-paper electrophoretic experiment was carried out in a cold room (1°), by the use of essentially the same apparatus as that employed by Grassman and Hannig (*6*). Peroxidase fraction on the filter-paper was detected by the use of guaiacol-hydrogen peroxide mixture according to the method of Jermyn and

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* A part of this work was presented at the "Symposium on Enzyme Chemistry" held in Sapporo, Hokkaido, in July, 1958.

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Thomas (*5*). They used 'positive' filter-paper strips treated with HIO_4 and Girard reagent, but untreated filter-paper strips (Toyo filter-paper No. 51) were used in the present experiments. The overall length was 36 cm. between the fluid levels, and the horizontal portion was of 30 cm. length. The neutral point was detected by the use of trinitrobenzene and dimethylaniline (*7*).

Zone Electrophoresis—The apparatus used was essentially the same as that described by Kunkel and Slater (*8*). The experiment was carried out by the use of a large trough (40×10×1.5 cm.) in a cold room at 1°, for 20 to 48 hours, applying a current of 15 to 20 mA. Prior to electrophoresis, peroxidase preparation was completely dialyzed against distilled water and then against acetate buffer (pH 4.6, ionic strength 0.1), and finally its protein concentration was adjusted with the same buffer to about 5 per cent.

Boundary Electrophoresis—Electrophoretic experiments were carried out at 5° in a Tiselius-type apparatus, using a microcell of 2 ml.-capacity. Prior to electrophoresis, each preparation was dissolved in a buffer of desired pH and dialyzed against the same buffer. Mobility, reduced to 5°, was calculated for the descending boundary using the value of specific electric conductivity measured at 5°.

Sedimentation—Sedimentation analysis was performed in a Spinco model E analytical ultracentrifuge. Prior to ultracentrifugation, each preparation was dissolved in a phosphate buffer of pH 7.0 and dialyzed against the same buffer. The ionic strength of the solution was finally adjusted to 0.2 by the addition of sodium chloride solution. Assuming the partial specific volume of the protein to be 0.75 ml. g^{-1}, the sedimentation coefficient was calculated, and corrected to standard conditions by the method rcecently summarized by Schachman (*9*).

Light Absorption—The absorption spectrum was measured using a Beckman model DU spectrophotometer, and plotted in terms of molar extinction coefficient of hemin. Hemin concentration was estimated by the method of Keilin and Hartree (*10*) employing the molar extinction coefficient of pyridine hemochromogen determined by Paul *et al.* (*11*), ($34.7\times10^3\,M^{-1}\,cm.^{-1}$ at 557 mμ).

Molecular Weight—The minimum molecular weight was determined from the measurements of the dry weight and the hemin concentration of the dialyzed solution of each preparation, by the method of Keilin and Hartree (*10*).

Purification Procedure

Step 1: *Crude Extract*—543 kg. of well-washed roots were used as the starting material. The roots were grated mechanically and suspended in water (about 2 liters of water was used per 10 kg. of the roots). The suspension was squeezed through cotton cloth. About 500 liters of juice was obtained.

Step 2: *First Ammonium Sulfate Fractionation*—The juice was brought to 80 per cent saturation[1] of ammonium sulfate and the mixture was left standing overnight. The fluid was filtered off through large fluted filters, the residue was suspended in twice its volume of water, and mixed thoroughly. The suspension was filtered through fluted filters, giving a reddish-brown filtrate. The residue was collected and extracted twice with an equal volume of water. (The degree of saturation of the ammonium sulfate in the suspension was adjusted to about 30 per cent by the addition of an appropriate amount of solid ammonium sulfate.) All extracts were combined and brought to 80 per cent saturation of ammonium sulfate. The precipitate obtained was suspended in a minimum amount of water, dialyzed against water, and filtered, yielding a dark reddish filtrate (F_1).

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[1] Unless otherwise stated, the degree of saturation of ammonium sulfate was accomplished by the addition of solid ammonium sulfate according to the Table of Green and Hughes (*12*).

Step 3: *Second Ammonium Sulfate Fractionation*—The filtrate F_1 thus obtained was fractionated by the addition of solid ammonium sulfate into two fractions corresponding to 0 to 50 per cent saturation (Fraction II) and 50 to 80 per cent saturation (Fraction I). Each fraction was dialyzed against water and, after filtration, the clear filtrates F_2 and F_3 were obtained for Fraction I and II, respectively. The filtrate F_3 was dialyzed, and re-fractionated by the addition of saturated ammonium sulfate solution into the fractions of 0 to 30 per cent and 30 to 50 per cent saturation, the former being discarded. The latter fraction was dialyzed and filtered, giving a filtrate (F_4).

Step 4: *Ethanol Fractionation*[2)]—Fraction I: The filtrate F_2 was chilled to 0° and an equal volume of cold ethanol was added with mechanical stirring, lowering the temperature of the mixture progressively to $-10°$. After 30 minutes, the fluid was centrifuged, giving a supernatant (S_A-1, Fraction A) and a viscous precipitate (P_B, Fraction B). Fraction II: The filtrate F_4 was dialyzed and treated with ethanol by the same method as Fraction I. By centrifugation of the mixture, a supernatant (S_C, Fraction C) and a precipitate (P_D, Fraction D) were obtained.

Step 5–1: *Purification of Fraction A*[3)]—A small amount of alumina gel[4)] suspended in an equal volume of ethanol ($-10°$) was added to the supernatant S_A-1 with mechanical stirring and the suspension was left standing for several hours. After centrifugation, the same procedure was repeated with the supernatant until the reddish color of the supernatant disappeared. The precipitates obtained were all combined and the adsorbed peroxidase was extracted several times with a small quantity of water. The extracts were all combined to give 1.8 liters of solution. The solution was again similarly treated with ethanol and alumina gel and the cake obtained (150 ml.) was eluted several times with a small quantity of 0.25 M disodium phosphate solution. The eluates with R. Z.[5)] >1.7 were all combined to give 500 ml. solution and brought to 80 per cent saturation of ammonium sulfate. After centrifugation, the precipitate was dissolved in a small quantity of water and dialyzed. After centrifugation, the resulting supernatant (S_A-2) was sub-

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[2)] On all occasions where ethanol was present, the liquids and centrifuge tubeswer e cooled in a freezing mixture, but the use of a refrigerated centrifuge was found to be unnecessary.

[3)] The purification procedure with ethanol and alumina gel for this Fraction A followed the method of Yamazaki (*4*).

[4)] Alumina gel used was ortho-aluminium hydroxyde prepared by the α-form method (*13*) and preserved in a refrigerator. Its adsorption ability did not seem to change at least for several weeks, though the change from α to β or γ from may occur.

[5)] See footnote *b* in Table II.

mitted to zone electrophoresis on starch in an acetate buffer (pH 4.6, ionic strength 0.1). Two main components of the peroxidase were observed, one (designated as "turnip peroxidase A_1") of which migrated toward the anode and the other (designated as "turnip peroxidase A_2") migrated toward the cathode, although other faint bands were sometimes observed. The starch segments of these two components were separately extracted with water. The extracts were brought to 80 per cent saturation of ammonium sulfate and the precipitates thereby obtained were separately dissolved in a minimum amount of water in a small stoppered test tube. Then the procedure of crystallization for these components was performed according to the method of Kenten and Mann (*14*), with slight modifications. The content of the tube became gel-like due to the occurrence of irregularly shaped microscopic particles, but attempts to obtain a fine crystalline form were unsuccessful. After centrifugation, the precipitate was dissolved in water and the solution was used for various tests. The R. Z. of the solution of turnip peroxidase A_1 was found to be 3.36 and that of A_2 to be 3.73.

Step 5-2: *Purification of Fraction B*—The precipitate P_B was suspended in an equal volume of water and mixed thoroughly. Insoluble matter in the mixture was removed by centrifugation and the supernatant was treated with ethanol by the same method as described in Step 4. After centrifugation, the supernatant was subsequently combined with Fraction A, while the precipitate was again extracted with an equal volume of water. The extract (S_B) was brought to 80 per cent saturation of ammonium sulfate and the precipitate collected by a centrifuge was dialyzed and submitted to zone electrophoresis by the same method as described above. A component that migrated toward the cathode at a high rate was detected, together with some faint bands. The starch segment of the main component (designated as "turnip peroxidase B") was extracted with water and submitted to various tests.

Step 5-3: *Purification of Fraction C*—Since this fraction was small in volume and found to be essentially a mixture of components of other fractions, description of further purification is omitted.

Step 5-4: *Purification of Fraction D*—The precipitate P_D, about 500 ml., was suspended in an equal volume of water. Insoluble matter in the mixture was removed by centrifugation. The supernatant (S_D), after dialysis, was brought to 80 per cent saturation of ammonium sulfate and the resultant precipitate was collected by centrifugation, suspended in a minimum quantity of water, and dialyzed. After centrifugation, the supernatant was submitted to zone electrophoresis as described above. The main component, which moved toward the cathode at a rate lower than that of turnip peroxidase B but higher than that of turnip peroxidase A_2, was extracted with water. By the same procedure of crystallization as described in Step 5-1, fine needle crystals were obtained as shown in Fig. 1 and named "turnip peroxidase D".

Purpurogallin Number (*P. Z.*) *of the Purified Preparations*—Activity of the four preparations thus obtained was determined through the purpurogallin test

TABLE I

Fractionation of Multi-components in Turnip Peroxidase

	Fraction	Ammonium sulfate fractionation	Ethanol fractionation a)	Rate of migration in zone electrophoresis at pH 4.6 b)
		%	%	cm. per hour
I	A_1	50—80	>50	−0.2
	A_2	50—80	>50	+0.15
	B	50—80	<50	+1.0
II	C	30—50	>50	—
	D	30—50	<50	+0.5

a) '<50' or '>50' signify that the fraction is precipitated or not precipitated by 50% ethanol solution.

b) Roughly estimated. Plus sign signifies migration toward the cathode, and minus, toward the anode.

TABLE II

Purification of Turnip Peroxidase

Fraction	Volume	A_{405} a)	R.Z. b)	Haemin content c)	Peroxidase content d)	Peroxidase unit e)	P.Z. f)
	ml.			μ mole	mg.		
juice	506400					125×10^5	
F_1	6580	10	0.20	660	30000		
F_2	770	19.8	0.55	152	6800	2.9×10^5	
F_3	720	13.5	0.32	97	4400	15.0×10^5	
S_A-2	200	25.8	1.90	52	2400		
S_B	400	28.3	0.50	113	5100		
S_D	500	13.3	0.43	67	3000		
TP-A_1			3.36		30		340
TP-A_2			3.73		40		260
TP-B			1.35		70		(300)
TP-D			2.46		35		1200

a) Absorbance at 405 mμ

b) Ratio of the absorbance at 405 mμ to that at 275 mμ, except in the case of the last four purified preparations, for which the ratio of absorbance at exact maximum wavelength of Soret band to that at exact maximum (or shoulder) wavelength of protein band is given.

c) Assuming molar extinction coefficient at 405 mμ of heme protein to be all 10^5.

d) Assuming no other heme protein than peroxidase to be present and the molecular weight of the peroxidase to be 45,000, except in the case of four finally purified preparations.

e) One unit of peroxidase activity was defined by the present author as a change in absorbance of 1.0 per second at 470 mμ under the conditions described below. The over-all reaction was followed by the Beckman spectrophotometer at 30° by observing the change in light absorption at 470 mμ due to the formation of dihydroxy-dimethoxy diphenyl. Quartz cells of 1 cm. contained 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.0), 2.0 ml. of 0.0172 *M* guaiacol, 0.1 ml. of enzyme solution. The reaction was initiated by the addition of 0.027 ml. of 2.0 *M* hydrogen peroxide and it was recorded at 10-second intervals, then the initial rate was determined. The enzyme solution was adjusted to give the change of absorbance of below 0.01 per second. (An optimum condition was found to be approximately attained in the concentrations mentioned above of guaiacol and hydrogen peroxide).

f) Purpurogallin number measured by the method of Keilin and Hartree (*10*).

(*10*). As seen in Table II, the P. Z. of TP-A_1[6] and A_2[6] were considerably low, while the value of TP-D[6], 1200, was comparable to that of HRP-II[6] (*10*), 1220, and that JRP-*a*[6] (*15*), 1420.

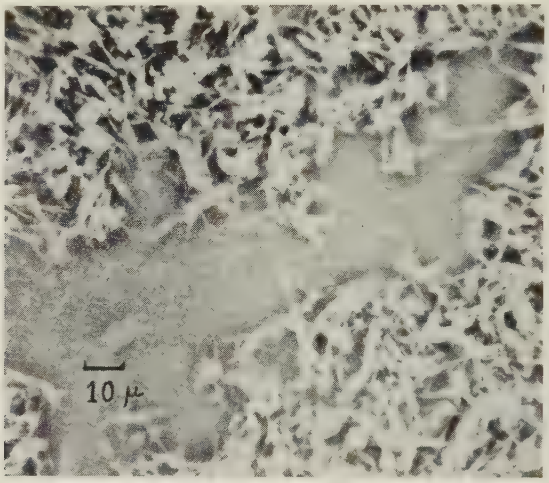

FIG. 1. Microphotograph of crystalline turnip peroxidase D. Phase contrast.

Physicochemical Properties

Filter-paper Electrophoresis -By filter-paper electrophoresis which was carried out in an acetate buffer of pH 4.6, ionic strength 0.1, at least three kinds of

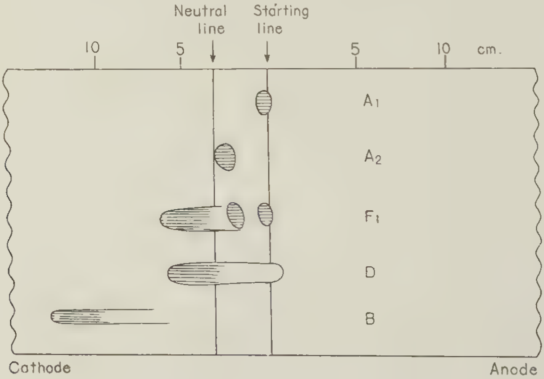

FIG. 2. Paper electrophoretic pattern of turnip peroxidases. 0.3 mA per cm. width, 36 hours, in acetate buffer (pH 4.6, ionic strength 0.1). F_1: crude preparation of Step 2; A_1, A_2, B and D: finally obtained preparations of each fraction.

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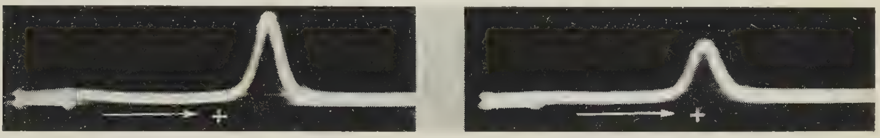
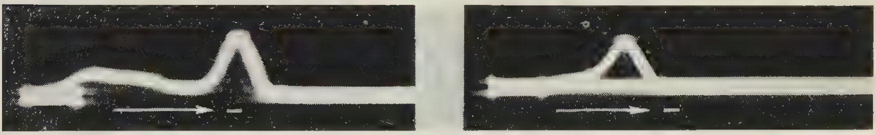
[6] TP-A_1, A_2, B, and D stand for turnip peroxidases A_1, A_2, B, and D, respectively. HRP-I, II and JRP-*a*, *c* stand for horseradish peroxidases I and II, and Japanese-radish peroxidases *a* and *c*, respectively.

peroxidases were found to be contained in the filtrate F_1, the crude preparation. In order to examine whether the finally purified preparations, namely TP-A_1, A_2, B and D, correspond to each component of the filtrate F_1 or not, they were submitted to electrophoresis on a single strip of filter-paper together with F_1 as shown in Fig. 2. From the result, it may be concluded that TP-A_1, A_2 and D correspond to each of three components of F_1, whereas no component corresponding to TP-B is present in the spots of F_1. Hence the fraction of TP-B may be a substance formed secondarily during the course of isolation.

Boundary Electrophoresis—Concurrently with the filter-paper electrophoresis, studies were also made by the moving-boundary method. It was found that TP-A_1 A_2 and D showed only a single peak, at least under the experimental conditions of Fig. 3, whereas TP-B contained slower components besides a main one. The calculated mobilities of TP-A_1, A_2 and D as well as the main peak of TP-B are presented in Table III.

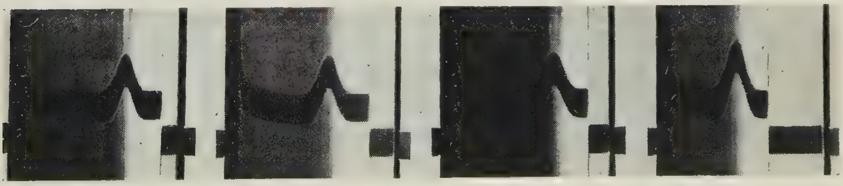


(a) Turnip peroxidase A_1 (b) Turnip peroxidase A_2



(c) Turnip peroxidase B (d) Turnip peroxidase D

FIG. 3. Electrophoretic patterns (ascending) of four kinds of turnip peroxidases.
(a), (b) and (c): In phosphate buffer of pH 7.1, ionic strength 0.2, 9000, 10800 and 9600 sec., 10 mA.
(d): In acetate buffer of pH 5.4, ionic strength 0.1, 7200 sec., 6 mA.



(a) Turnip peroxidase A_1 (b) Turnip peroxidase A_2 (c) Turnip peroxidase B (d) Turnip peroxidase D

FIG. 4. Sedimentation patterns of four kinds of turnip peroxidases in phosphate buffer of pH 7.0 at ionic strength of 0.2.
(a), (b) and (c): After 64 min. at 59,820 r.p.m.
(d): After 48 min. at 59,880 r.p.m.

Sedimentation—Sedimentation studies of TP-A_1, A_2 and D provided an additional evidence of homogeneity as shown in Fig. 4. The fraction of TP-B, however, was found to contain faster components in addition to a main one. The values of sedimentation coefficient $s_{20,w}$ of TP-A_1, A_2 and D as well as the main component of TP-B are given in Table III.

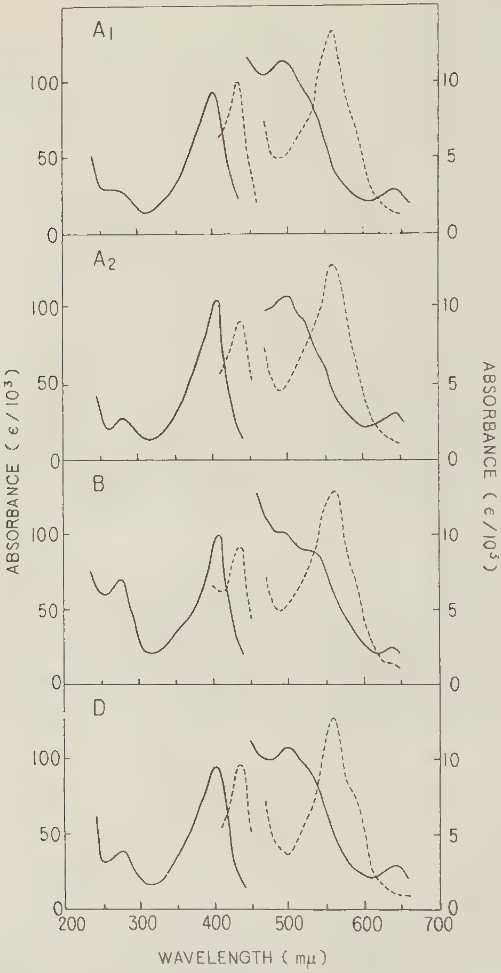


FIG. 5. Absorption curves of four kinds of turnip peroxidases and of their reduced forms.
Solid curve: native form; broken curve: reduced form.
A_1, A_2, B and D: turnip peroxidases A_1, A_2, B and D, respectively.

Absorption Spectra and Reinheit Zahl—Fig. 5 shows the absorption curves of native and reduced peroxidases. The values of wavelength of absorption maxima and corresponding molar extinction coefficients are presented in Table III, together with R. Z. calculated from the values. Besides, complex II of

TABLE III

Physicochemical Properties of Turnip Peroxidases and Other Crystalline Phyto-peroxidases

	The present author's				Other workers'		
	TP-A_1	TP-A_2	TP-B	TP-D	HRP-II[a]	JRP-a[b]	TP[c]
Absorption Maxima[d]							
Native peroxidase pH 7.0	276^s(27.8) 402 (93.6) 494 (11.4) 643 (2.86)	277^s(28.4) 405 (106.0) 499 (10.9) 643 (3.13)	277 (70.4) 406 (100.0)[e] 492 (10.2)[e] 533 (8.78)[e] 635 (2.50)	278 (38.4) 404 (94.5) 497 (10.9) 642 (2.95)	403 (89.5) 500 (9.9) 640 (2.7)	276 (33.8) 405 (111.0) 500 (11.75) 640 (3.27)	403 (90)
Reduced peroxidase[f]	436 (98.8) 559 (13.4)	438 (91.5) 559 (13.1)	437 (91.9) 559 (13.0)	438 (96.5) 557 (12.8)	438 (74.5) 554 (8.5)	439 561	
Complex II[g]	420	420	420	420	418		
R.Z.	3.36	3.73	1.35	2.46	3.0	3.28	2.7
Molecular Weight[h]	49,000	45,000	(65,000)	43,000	40,200	54,500	
$s_{20,w}$[i]	3.41	3.31	3.17	3.21	3.59		
$u \times 10^5$ cm.^{2} volt^{-1} sec.$^{-1}$ pH 7.10	-2.8	-1.8	+2.0	~0		-1.98 (pH 7.17, $\mu=0.1$)	
pH 5.42				+1.8			

a) Horseradish peroxidase II (*3*)
b) Japanese-radish peroxidase *a* (*15*)
c) Turnip peroxidase (*4*)
d) Wavelength in millimicrons, and corresponding millimolar extinction coefficients in parenthesis. 's' signifies shoulder.
e) The maximum wavelengths of absorption spectrum, observed immediately after the segment of starch was extracted with water, were 420 and 530 mμ. The spectrum was so unstable that it changed even during the measurement. The values in this table were obtained after precipitation with ammonium sulfate.
f) By addition of $Na_2S_2O_4$ (*10*).
g) By the method of George (*16*).
h) From chemical analysis except in the case of horseradish peroxidase II.
i) In terms of Svedberg unit.

these peroxidases was observed by the method of George (*16*) and the values of wevelength of absorption maxima are also presented in Table III. For the purpose of comparison, the corresponding values of HRP-II (*3*), JRP-*a* (*15*) and the crystalline turnip peroxidase obtained by Yamazaki (*4*) are also listed in Table III. This table shows clearly that the absorption maxima of these peroxidases agree very closely with each other.[7] Furthermore, on treatment with pyridine and sodium hydrosulfite (*10*), these four preparations gave the same hemochromogen as that of horseradish peroxidase II. It appears that all these turnip peroxidases have the same prosthetic group as that of HRP-II and that of JRP-*a*, namely protohematin.

Molecular Weight—The values given in Table III are the minimum molecular weight of each peroxidase determined from the measurement of dry weight and hemin concentration. At least in the case of TP-D, the minimum molecular weight was found to agree with the molecular weight determined on the basis of diffusion and sedimentation coefficients, namely 41500[8]. Accordingly, it is concluded that TP-D possesses only one prosthetic group in a molecule. Although diffusion coefficients of TP-A_1 and A_2 are not yet obtained and accordingly those true molecular weights are obscure, it is very likely that these enzymes may also possess only one prosthetic group in a molecule.

DISCUSSION

From the results presented above, it is concluded that the three of four finally obtained preparations, namely TP-A_1, A_2 and D, are native and pure peroxidases. These peroxidases had the same prosthetic group and a similar molecular weight, but they were quite different from each other on the points of behaviour in electrophoresis, and solubility in ammonium sulfate solution and in aqueous ethanol. From these results, it is suggested that some properties of their protein moiety, for instance, the kind and state of ionic groups on the surface of the proteins, are different from each other. The difference in the activity of these peroxidases expressed as the values of P. Z. or the rate constant, k_d[9], may be due to that in the protein moiety of these components described above.

It is remarkable that the P. Z. of TP-A_1 and A_2 were considerably lower than that of TP-D, in spite of their highest values of R.Z. The R.Z. of TP-D was the lowest of these preparations, but this enzyme was of high purity and had high values of P.Z. and the rate constant, k_d[9], for guaiacol. These facts show that P. Z. and R. Z. are not necessarily a suitable measure for representing and comparing the purity of peroxidases obtained from various kinds of plants as has been considered so far.

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[7] However, only in the case of TP-B, an abnormal phenomenon was observed as described in footnote *e* in Table III.

[8] To be published in detail in a succeeding paper.

[9] The data on the rate constant, k_d, will be described in a succeeding paper.

Another turnip peroxidase, TP-B, originally exhibited the absorption spectrum with maxima at 420 and 530 mμ. It is of interest to note that this absorption spectrum is similar to that of JRP-*c*[6] (419, 540 mμ) (*17*) and that of HRP-I[6] (420, 540 mμ) (*18*). According to Morita and Kameda (*17*), JRP-*c* (*17*) is a native peroxidase contained naturally in Japanese-radish roots. Keilin and Hartree (*10*) insisted that HRP-I is a transformation product of true peroxidase, HRP-II, but Jermyn and Thomas (*5*) supposed it to be a native one in view of the results obtained by the use of filter-paper electrophoresis. As previously mentioned, the present author supposed the component of TP-B to be an artifact which had a characteristic property of easily changing its absorption spectrum.

There are apparently some discrepancies between the results of the present work and those obtained using turnip peroxidases extracted from other kinds of turnip roots. According to Jermyn and Thomas (*5*), only two components were found to be contained in the juice of their turnip roots by means of filter-paper electrophoresis, in contrast to three components found in the present experiment. And, Yamazaki (*4*) reported that a crystalline turnip peroxidase was able to be obtained from the fraction corresponding to Fraction A, using turnip roots gathered in June in Hokkaido (the name of the variety and the activity are obscure), though in our experiments it was unsuccessful to obtain a crystalline peroxidase from Fraction A. The present author also could, on another occasion, obtain easily a crystalline turnip peroxidase from the corresponding fraction of a different source (the variety of "Shimokaburi" gathered in January in Tokyo) and found that the crystalline turnip peroxidase had the activity higher than that of TP-A_1 and A_2 but lower than that of TP-D (unpublished data).

These discrepancies may be due to the difference in varieties of plants, but the possibility may not be excluded that they are due to seasonable variation of turnip peroxidase, in view of the fact that such seasonable variation was observed in the case of HRP (*5*). In order to clarify this problem, further detailed studies must be carried out. At any rate, in order to obtain reproducible results, it would be necessary to use a fixed source, that is, the turnip of a definite variety gathered in a definite season. The reproducibility has been confirmed at least in the case of the turnip used in our studies.

SUMMARY

1. It was found by means of filter-paper electrophoresis that at least three kinds of peroxidases are present in the crude extract of turnip roots used in the present experiment.

2. These three peroxidases were isolated separately and highly purified, and designated as "turnip peroxidases A_1, A_2 and D". Turnip peroxidase D was obtained in a crystalline form (needle shape).

3. Physicochemical properties of these preparations were examined and thier homogeneity was confirmed. All these peroxidases obtained had the same prosthetic group as that of horseradish peroxidase, and their molecular

weights or sedimentation coefficients were found almost same each other. They exhibited, however, different behaviour in electrophoresis.

4. The P. Z. of turnip peroxidase D was comparable to that of horseradish peroxidase II or that of Japanese-radish peroxidase *a*, while the P. Z. of turnip perosidases A_1 and A_2, in spite of the highest R.Z. of these enzymes, were considerably lower than that of turnip peroxidase D.

5. Besides the above peroxidases (TP-A_1, A_2 and D), another peroxidase having the absorption spectrum similar to that of horseradish peroxidase I was obtained. However, this peroxidase was considered to be a substance produced during the course of isolation.

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STUDIES ON CYTOCHROME A

V. PROPERTIES OF COPPER IN PURIFIED CYTOCHROME A*

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In a previous communication (*1*), Okunuki *et al.* reported that their purified preparation of cytochrome a contained not only heme-iron but also copper. Eichel *et al.* (*2*) and Green and Crane (*3*) have reported that their cytochrome oxidase preparations contain a high concentration of copper as well as heme-iron. Moreover, many investigators (*4*, *5*, *6*) have observed that a specific deficiency of cytochrome a and a conspicuous decrease in cytochrome oxidase activity are caused by a copper-deficient diet but restored to normal by administration of copper. The above and other facts suggest that the copper in cytochrome oxidase plays an important role in its reaction mechanism. In fact, it is well known that many copper enzymes exhibit the oxidase reaction in the presence of molecular oxygen and various substrates (*e.g.* tyrosinase or laccase). In the case of cytochrome a, there was no direct evidence to support the above role of copper. In the present communication detailed results are given of metal analysis and some of the properties of the copper in a purified cytochrome a. It is reported that copper changes in valency with the valency change of heme-iron during the cytochrome oxidase reaction. A part of this investigation has been already reported (*7*).

EXPERIMENTAL

Materials—Cytochrome a was prepared according to the method described in the previous communication (*1*) except that it was not treated with Cγ alumina gel. To avoid contamination by copper, copper was carefully removed from the water used for extraction and purification of the cytochrome a. Crystalline oxidized cytochrome c was prepared from beef heart by the method of Hagihara *et al.* (8). The crystals were dissolved in 0.1 *M* phosphate buffer (pH 7.4) and used after dialysis against the same buffer for 4 hours. Lactic dehydrogenase was prepared from baker's yeast according to the method of Yamanaka *et al.* (*9*). Emasol 4130, a non-ionic detergent from Kao Soap Co., was used after dilution to 10 per cent (v/v) with 0.1 *M* phosphate buffer, pH 7.4. Other materials used were the same as previously described (*1*, *7*).

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* This paper was reported at the 11th Meeting of Enzyme Chemistry, at Kyoto, Japan, on July 20, 1959 (*13*).

This investigation was supported in part by the Grant RG-5871 from National Institutes of Health, U. S. A.

Methods—The concentration of cytochrome a was determined spectrophotometrically from the difference in light absorption at 605 mμ and 630 mμ, using the value of $16.5\times 10^3\ M^{-1}.\ \mathrm{cm}^{-1}$ which was calculated from the results given in the 3rd communication of this series (*14*). The concentration of protein was expressed as protein-nitrogen as measured by the micro-Kjeldahl method. Iron was determined with *o*-phenanthroline using a modification of the method of Sandell (*11*). Copper was determined colorimetrically by the diethyldithiocarbamate method using amyl alcohol as solvent (*12*). Spectrophotometric determinations were made in a Cary spectrophotometer Model 14 and Shimadzu spectrophotometer type QB-50 with cells of 1 cm. light-path. The cytochrome oxidase activity was measured manometrically in a Warburg apparatus as described previously (*14*). The valency state of copper was determined by a modification of the methods of Hoste (*16*) and Smith and McCurdy (*17*) as follows; A sample of cytochrome a was diluted to a suitable concentration with 0.1 *M* phosphate buffer (pH 7.4) containing 1 per cent Emasol 4130. One milliliter of glacial acetic acid containing 1 mg. of 2,2′-diquinolyl or 2,9-dimethyl-1,10-phenanthroline (neocuproine) was pipetted directly into a cell which contained 2 ml. of cytochrome a solution. The characteristic color of the cuprous copper was measured spectrophotometrically at 540 mμ for 2,2′-diquinolyl or at 450 mμ for neocuproine. For anaerobic incubations, Thunberg tube type cells were used.

RESULTS

1) *Copper Content of a Highly Purified Cytochrome a*—In a previous communication (*1*), it was reported that a cytochrome a preparation contains copper in an amount such that the molar ratio of copper to heme-iron is 4.5. Later, the copper content of various preparations were repeatedly analysed and it was found that about 80 per cent of the total copper can be removed from the protein moiety by dialysis against a copper chelating agent. There was no loss of the oxidase activity after the complete removal of the reagent by redialysis of the preparation against 0.1 *M* phosphate buffer containing 0.5 per cent Emasol 4130 at pH 7.4. From the above results it seems likely that a large part of the total copper is not essential for oxidase activity, while the rest of the copper is combined with the protein moiety so strongly that it can not be detached during purification. To determine whether this is true, cytochrome a was carefully prepared using chemical reagents and water from which copper had been completely removed. The copper content of the preparation thus obtained was assayed. It is obvious from Table I that the copper content decreased to one atom per one atom of iron or per mole of cytochrome a.

The relation between the contents of cytochrome a and copper during the purification process is also illustrated in Table II. As can be seen in Table II, there is at least one atom of copper per mole of cytochrome a at all of the purification stages, although the copper content of these fractions is not proportional to the oxidase activity. Thus, the relation between cytochrome oxidase activity and the metal content is rather obscure in contrast to that of other metal containing enzymes. The decrease in oxidase activity in Fractions S_3 and S_4 will be discussed later in this paper.

2) *Effect of Copper-Chelating Agents*—The cytochrome oxidase activity of

this preparation was inhibited by usual heme-iron binding inhibitors such as cyanide, azide and carbon monoxide, as reported previously (*14*). Moreover, about 50 per cent of the activity was lost by treatment with copper-chelating agents such as ferrocyanide, salicylaldoxime and ethylxanthate as illustrated in Table III. Other copper binding agents such as diethyldithiocarbamate, and phenylthiourea and iron-binding agents were tested at a concentration of

TABLE I

Copper and Iron Contents of Fraction S_4 of Cytochrome a

Preparation No.	Content (μM × 10^{-2} per mg. protein N)			Cu/Fe	Cu/Cyt. a
	Cu	Fe	Cyt. a		
1	6.35	6.07	5.65	1.05	1.12
2	5.68	5.65	4.91	1.01	1.16
3	5.91	5.65	5.36	1.04	1.10
4	5.58	4.89	4.23	1.10	1.32
5	6.27	5.95	5.09	1.05	1.23
Average	5.96	5.64	5.05	1.05	1.18

TABLE II

Relation between Copper and Cytochrome a Contents during the Purification Process

Stage of Purification[1]	S_1	S_2	S_3	S_4
Turnover number (μM O_2/μM Cyt. a/min.)	114.2	150.0	42.2	36.4
Cytochrome a (μM × 10^{-2} per mg. N)	1.05	1.07	3.75	5.09
Copper (μM × 10^{-2} per mg. N)	1.10	1.43	4.76	6.09
Copper/Cytochrome a	1.04	1.33	1.27	1.19

1) The nomenclature of the fractions is the same to that in a previous report (*1*).

TABLE III

Effect of Chelating Agents on the Activity of Cytochrome a

Reagent	Concentration (*M*)	Inhibition (%)
KCN	10^{-5}	60
NaN_3	10^{-3}	72
$K_4Fe(CN)_6$	10^{-2}	50
Salicylaldoxime	10^{-2}	50
Ethylxanthate	10^{-2}	58

10^{-2} *M*. No inhibition was observed. The sensitivity of the activity of cytochrome a to various chelating agents suggests that the copper directly participates in the cytochrome oxidase reaction.

3) *Nature of the Binding of Copper to Cytochrome a*—Hemin analysis by the acid-acetone method showed that more than 90 per cent of the total iron is accounted for as heme-iron, while more than 90 per cent of the total copper is recovered in the protein moiety of the preparation. The copper is quantitatively liberated as inorganic copper ion by treatment with trichloroacetic acid. It is not liberated by heat treatment, even at 100° for 30 minutes. These observations indicate that the copper is exclusively associated with the protein moiety of cytochrome a and not with heme part which contains iron. To prove that the copper acts as one of prosthetic groups of cytochrome a, an attempt was made to remove the copper from the protein moiety by dialysis against copper-chelating agents. However, it was not released by dialysis against cyanide or salicylaldoxime and the activity remained unaffected by the treatment as illustrated in Table IV. After the above treatment the activity can yet be inhibited by copper chelating-agents at the same concentration as given above. Even by repeated dialysis of reduced cytochrome a, no liberation of copper was observed. Thus all attempts failed to remove the copper completely. The copper seems to be firmly bound to cytochrome a.

TABLE IV

Effect of Dialysis on the Copper Content and Cytochrome Oxidase Activity of Cytochrome a

Dialysis solution	Copper content (γ/mg. N)	Cytochrome oxidase activity (O_2 μl./10 min.)
(A) 0.1 *M* phosphate buffer (pH 7.4)+1% Emasol 4130	2.96	52
(B) (A)+10^{-2} *M* KCN	2.59	38
(C) (A)+10^{-3} *M* Salicylaldoxime	2.90	52

10 ml. of 5.8×10^{-5} *M* cytochrome a were dialyzed against 500 ml. of a solution of each chelating agent for 48 hours at 5°. These samples were then re-dialyzed against 1000 ml. of 0.1 *M* phosphate buffer (pH 7.4) containing 0.5 per cent Emasol 4130 for 60 hours at 5°. The dialysis water was changed six times.

The copper content is given as a difference between the copper in cytochrome a and that in an equal volume of the surrounding solution.

4) *Ultraviolet Absorption Spectrum of Cytochrome a*—The absorption spectrum of purified cytochrome a shows two distinct peaks at 424 mμ and at 600 mμ in the oxidized form and these are attributed to heme a. The ultraviolet absorption spectrum of oxidized cytochrome a shows a broad band at 340 mμ in addition to the protein band at 280 mμ as shown in Fig. 1.

The optical density of the 340 mμ peak is somewhat decreased by dialysis against cyanide. Heme a which was liberated by the acid-acetone method shows no peak in this wavelength. It is of interest that the complex of cupric ion with albumin (*15*) and other copper enzymes exhibit an absorption band in this wavelength. However, there is no direct evidence that the peak at 340 mμ is due to the combination of cytochrome a with copper.

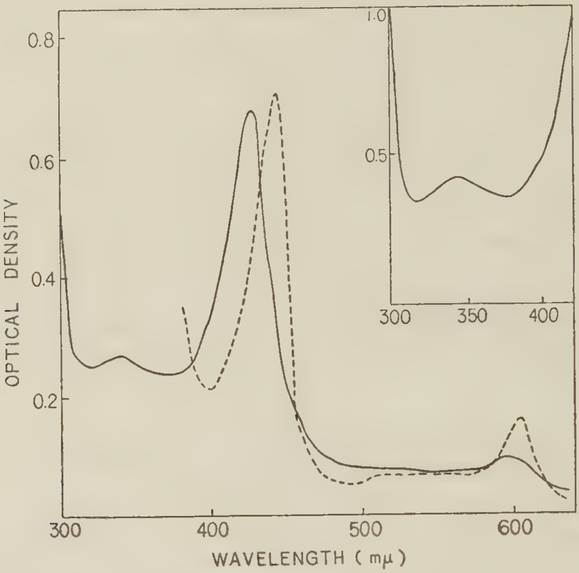

Fig. 1. Absorption spectra of cytochrome a.

Visible spectrum is shown on large graph and ultraviolet range in insert. The spectra were determined in 0.1 *M* phosphate buffer (pH 7.4) containing 1.0 per cent Emasol 4130. The ' reduced ' spectrum was obtained by the addition of a small amount of sodium dithionite. Solid line, oxidized form; Dotted line, reduced form.

5) *Valency of the Copper*—The valency of the copper in cytochrome a was determined with 2,2′-diquinolyl or 2,9-dimethyl-1,10-phenanthroline (neocuproine). These are chelating-agents which are specific for cuprous copper and the cuprous ion is quantitatively measured by the color development of the cuprous chelating complex (*16*, *17*). The results of the reaction of copper in cytochrome a with the above reagents in glacial acetic acid are illustrated in Table V.

Addition of the reagents to cytochrome a in Emasol 4130 gives a strong specific color due to cuprous ion, showing that glacial acetic acid can release the copper efficiently and quantitatively from cytochrome a. However, as in the case of the iron in succinic dehydrogenase (*18*), a possibility existed that liberation of a reducing group from the protein could cause secondary reduction of cupric to cuprous ion. As illustrated in Table V, the addition of PCMB inhibits color development with cuprous ion. This suggests that the copper of cytochrome a is largely or entirely in the cupric form. With increasing amount of PCMB, the color developed by the copper reacting as cuprous ion decreased and with $10^{-3}\,M$ PCMB there was hardly any color development. The color appeared on addition of a reducing agent (hydroxylamine) to the reaction mixture containing PCMB. Control experiments showed that under the conditions used for the determination, PCMB neither caused any oxidation of cuprous copper nor led to the liberation of protein groups which could oxidize cuprous copper.

TABLE V

Effect of p-Chloromercuribenzoate on Copper Analysis

Conditions	Per cent copper reacting as cuprous ion	
	Neocuproine	2,2′-Diquinolyl
(A) None	100	100
(B) $1.7\times10^{-3}\,M$ PCMB	0	20
(C) $1.7\times10^{-3}\,M$ PCMB $+0.3\%$ NH_2OH	117	120

The values were calculated from the optical density at 540 mμ (2,2′-diquinolyl) or at 450 mμ (neocuproine) 10 minutes after the addition of chelating agent.

TABLE VI

Effect of Substrate on Valency of Copper of Cytochrome a

Conditions	Per cent copper reacting as cuprous ion (%)
(A) $1.5\times10^{-5}\,M$ Cytochrome a	100
(B) (A)$+1.4\times10^{-3}\,M$ PCMB	7
(C) (B)$+2.4\times10^{-6}\,M$ Cytochrome c +Lactic dehydrogenase	2
(D) (C)$+7.0\times10^{-3}\,M$ Lactate (anaerobic)	100-80

Copper analysis was made with neocuproine. The values were calculated from the optical density at 450 mμ 10 minutes after the addition of reagent.

From the above results, it appears that the copper of cytochrome a may be present in the cupric state. If the copper of cytochrome a participated directly in the course of electron transfer from cytochrome c to oxygen, it would be reasonable to consider that changes in the valency of the copper might occur on addition of reduced cytochrome c. As shown in Table VI, the presence of substrates (lactate, lactic dehydrogenase and cytochrome c) under anaerobic conditions changed the valency of the copper. That is, the copper in reduced cytochrome a appeared to be in the cuprous form.

From the above results it seems that all or most of the copper in reduced cytochrome a is in the cuprous form and that of oxidized cytochrome a is in the cupric form.

DISCUSSION

The method which has been frequently used to implicate a metal in an enzyme protein is to demonstrate that a specific metal follows enzymatic activity throughout the process of purification. However, the copper of cytochrome a did not follow the oxidase activity. It can be seen that the oxidase activity of cytochrome a suddenly decreases during the purification step of Fractions S_3 and S_4. Such a decrease in the activity during purification does not seem to be due to the release of copper from cytochrome a, since the molar ratio of copper to cytochrome a was always one. Recently, Wainio *et al.* (*19*) and Hatefi (*20*) have reported the presence of a lipid-cofactor which reactivates the enzyme. From the above facts, it seems likely that the decline of oxidase activity is due to the separation of the lipid-like substance from cytochrome a during purification. This question is being studied. However, the facts described in the present communication strongly suggest that copper combines with the protein moiety of cytochrome a.

From the experimental evidence presented, it is likely that the copper in cytochrome a exists in the cupric form, and changes in valency during electron transfer from substrate to oxygen. Recently, such valency changes have been demonstrated with nitrate reductase of *Neurospora crassa* (*21*) and laccase (*22*). Possibly, more clear-cut evidence may be obtained by physical methods. The recent development of the electron spin resonance technique has made possible a more direct approach to this problem, and Malmström *et al.* (*23*) demonstrated by the electron spin resonance method that fungal laccase contains cupric copper which changes to the cuprous form during oxidation of the substrate. Therefore, until more extensive experiments are performed, the possible participation of copper in the cytochrome oxidase reaction must remain speculative. However, the experimental results presented here strongly support an importance of the role of copper in the cytochrome oxidase reaction. From a consideration of the redox potentials of copper and cytochrome a, it is reasonable to suppose that the copper reacts directly with molecular oxygen. On the other hand, the results presented in previous communications of this series (*10*, *14*) indicate that the heme-iron of cytochrome a can react with oxygen or carbon monoxide. It is noteworthy that the inhibition of the cytochrome oxidase activity of a cytochrome a preparation containing cytochrome c by carbon monoxide is light-sensitive. This discrepancy can only be explained by further investigations and the mechanism of cytochrome oxidase reaction will be better interpreted when these problems have been resolved.

SUMMARY

1. It was found that a purified preparation of cytochrome a contains

one atom of copper per one atom of iron or per mole of cytochrome a. Further, copper is presented at all of the stages of the purification in a quantity equal to at least one atom per mole of cytochrome a.

2. The cytochrome oxidase activity is inhibited by copper-chelating agents such as ferrocyanide, salicylaldoxime and ethylxanthate.

3. The copper in cytochrome a is not released from the protein moiety by dialysis against copper-chelating agents. Acid treatment of cytochrome a easily releases the copper from the protein moiety. These observations show that the copper is firmly associated with cytochrome a.

4. It appears that the copper in cytochrome a exists in the cupric state, and that changes in the valency of the copper occur on electron transfer from substrate to oxygen.

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RESPIRATION SUBSTRATE OF RHIZOBIUM IN THE NODULES

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Rhizobium is the symbiotic organism of root-nodules of leguminous plants. To study biochemical properties of the bacteria, it is important not only to use cells grown in artificial medium but also to use cells taken from root nodules, though in the latter case we may meet considerable technical difficulty. Symbiotic bacterial cells are considered to be adapted to special environments in the nodules. They are present in the protoplasm of the host cells, restricted in the supply of free oxygen (*1*), and surrounded by dense solution of hemoglobin (*2–4*) and riboflavin (*5*). The distinct biochemical characteristic of this organism is their nitrogen fixation. *Rhizobium* fixes molecular nitrogen only in symbiosis with the host probably via ammonia to glutamate, and then supplies it to the host plant roots. On the contrary the bacterial cells cultured outside of the host do not fix molecular nitrogen and need nitrogenous compounds such as amino acids, ammonia or nitrate for their growth. Terminal oxidases seem to be different between the two types of the cells (*6–7*). We shall report on the terminal oxidases in the next paper. Present paper is concerned with the substrates which the symbiotic bacterial cells receive for their respiration from the host plant.

It was found that symbiotic *Rhizobium* cells did not utilize glucose, fructose or sucrose, although these sugars are photosynthetically produced in host plants, and *Rhizobium* grows well on them when cultured in an artificial medium. So some members in the pathway of glycolysis such as hexose-phosphates and organic acids were studied whether they can serve as the substrate of respiration.

MATERIALS AND METHODS

1. Preparation of Resting Cell Suspensions of the Symbiotic Rhizobium Cells—The soybean bacterial strain (*Rhizobium japonicum*; Strain, Tôdai No. 53) was used throughout this experiment. The soybean plants (Nôrin No. 2) were grown in pots filled with sand. The bacterial strain was inoculated at the same time of seed sowing. Sand and seeds were not sterillized. Therefore, though large inoculum was used, it is not sure whether all of the bacteria in the nodules come from the inoculated strain. But the contamination of wild strains of *Rhiz. japonicum* does not interfere with the present conclusion. The plants were allowed to grow for 3 months. Then the nodules were detached from the roots, washed with running water, and disintegrated by a Waring blendor for half to one minute with twice volume of water. Debris of host plant tissue was filtered through cloth. The filtrate was centrifuged at 6,000 r.p.m. for 20 minutes and red supernatant was separated. The precipitated white paste was composed of bacterial cells, which are washed twice and suspended in $M/15$ phosphate buffer of pH 6.8, and stored in a refrigerater. Thirty ml. of this suspension was obtained from 15–20 g. of the nodules. The procedure was carried out in a cold room at about 2°. The bacterial suspensions were used for the experiments within 3 days, as prolonged storage depressed the oxidizing activity, especially the oxidation of substrates slowly oxidizable.

2. *Preparation of the Cells Grown on Glucose as Principal Carbon Source*—The resting cell suspensions of the same strain which is adapted to glucose were prepared as follows: The bacteria were subcultured from a stock culture of yeast extract-mannitol agar medium to a yeast extract-glucose agar slant. It was observed that the strain grew well on this medium. The bacteria were then inoculated in 10 ml. of yeast extract-glucose liquid medium and incubated at 30° for 48 hours in Monod-type shaking-culture tubes. The whole content of the tube was then transfered to 100 ml. of the medium in 500 ml. Sakaguchi flask and incubated on the shaker for 24–30 hours.

The yeast-glucose medium means the conventional yeast extract medium for *Rhizobium* where glucose was used instead of mannitol: Glucose 10 g., K_2HPO_4 0.5 g., $MgSO_4 \cdot 7H_20$ 0.2 g., NaCl 0.1 g., $CaCO_3$ 1.0 g., dried yeast extract (Daigo Eiyô Co.) 0.7 g., distilled water 1,000 ml., pH 7.0. Incubation time should not exceed 24–30 hours, because longer incubation made the culture slimy and difficult to get nice suspension, although the total crops of bacteria increased.

The cultures from several flasks were combined and centrifuged. Bacterial cells were washed and suspended in $M/15$ phosphate buffer of pH 6.8. About 30 ml. of this suspension of 1.6 mg./ml. dried cell was obtained from 5 flasks. The suspensions were not stored more than 6 hours before manometric measurements.

3. *Preparation of the Cells Grown on Succinate as Principal Carbon Source*—The procedures were the same as used for "the glucose cell" except that succinate was used instead of glucose. Synthetic medium was adopted to avoid possible contamination of sugar phosphate compounds from the yeast extract. The composition was as follows; Na-succinate 10 g., K_2HPO_4 0.5 g., $MgSO_4 \cdot 7H_2O$ 0.2 g., $CaSO_4 \cdot 2H_2O$ 0.2 g., NaCl 0.1 g., $FeCl_3$ trace, NH_4Cl 0.376 g., casein hydrolysate (Nissan-Suisan Lab., "Casamino acid") 100 mg., vitamin mixture 1 ml., distilled water 1,000 ml., pH 6.0. One mililiter of the vitamin mixture contained thiamine 1 mg., riboflavin 1 ml., Ca-pantothenate 1 mg., *p*-aminobenzoic acid 1 mg., niacin 1 mg., folic acid 100 μg., pyridoxine 50 μg., vitamin B_{12} 10 μg., biotin 2 μg.

4. *Measurements of Oxidizing Ability*—Oxidation of substrates was investigated by usual manometric technique. Total amount of reaction mixture in vessels was 2 ml., which contained about 20 mg. of the bacterial cells (as dry matter). The concetration of substrates and phosphate buffer were $M/20$ and $M/15$ respectively. Initial pH was 6.8. The substrates dissolved in $M/15$ phosphate was put in the side arm and was mixed at the proper time. Temperature was 30°. Each experiment involved at least two parallel measurements for each substrate, and the results agreed well. Average values are shown in Table 1.

The substrates tested were sugars which are those either commonly found in plants or customarily used for culture media (*1*), and members in the pathways of glycolysis (*2*), as well as members of Krebs cycle (*3*).

RESULTS

Oxidation of substrates by *Rhizobium* cells prepared by various methods is summarized in Table I. Oxygen uptakes are expressed by the value of O_2-uptake for 60 minutes per mg. dry matter, endogenous respiration being subtracted. Some results are also shown in Fig. 1-3. Oxygen uptake of the first 30 minutes was measured, because in this short period the synthesis of adaptive enzyme is supposed to be negligible.

TABLE I

Oxidation of Various Substrates
by Three Cell-Types of Rhizobium japonicum

Origin of the cell Substrate	Symbiotic cell	Glucose cell	Succinate cell
Glucose	0.64	6.60	1.64
Fructose	0.60		
Sucrose	0.50		
Mannose	0.60		
Galactose	0.32		
Mannitol	0.50		
G-1-P	0.06		0.20
G-6-P	0.16	0.80	4.60
F-6-P	0.28	0.26	3.56
F-1, 6-P	3.48	4.38	1.20
3-P-glycerate	0.24	0.30	0.58
P-enol-pyruvate	0.96		
Pyruvate	4.00	1.24	
Acetate	3.08		
Alanine	1.00		
Citrate	4.16		1.10
α-keto-glutarate	8.52	4.56	
Succinate	14.06	4.08	14.38
Fumarate	7.78	1.80	6.40
Malate	8.30		
Oxaloacetate	6.40		
Aspartate	5.10		
L-Glutamate	5.46		

Figures are Q_{O_2} in the first 30 minutes, and endogenous respirations were subtracted.

Endogenous respirations were
between 3.50–5.70, the average 4.5 for symbiotic cell
,, 3.26–3.34, the average 3.30 for glucose cell
3.08 for succinate cell

1. Oxidation of the Substrate by "Symbiotic Cell"—It became evident that glucose, fructose, sucrose, mannitol, galactose and mannose were scarcely oxidized by the symbiotic cells. On the contrary, all of the organic acids of

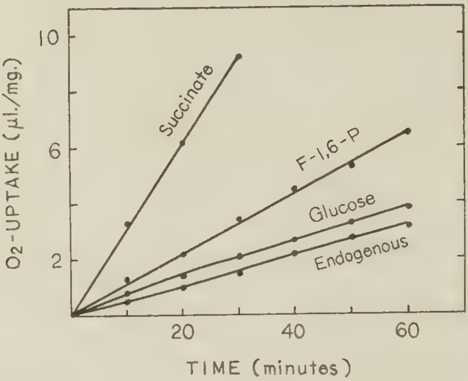

FIG. 1. Respiration of "symbiotic cell".

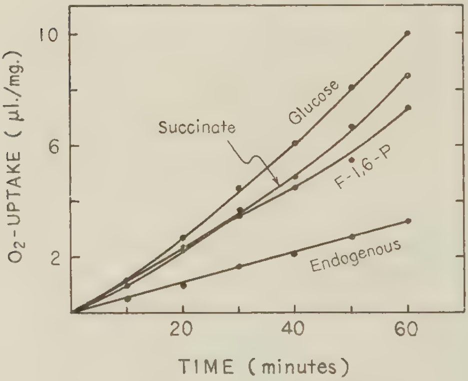

FIG. 2. Respiration of "glucose cell".

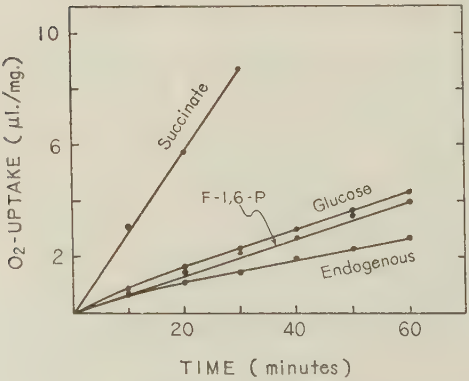

FIG. 3. Respiration of "succinate cell".

the Krebs cycle tested and also aspartate and glutamate were readily oxidized by these cells, and succinate oxidation was most rapid. Pyruvate and acetate were also oxidized by the cells, but alanine was only slowly oxidized, although it was found in soybean nodules. (*8*).

Among the intermediates* of glycolysis, only fructose-1,6-diphosphate** was oxidized, but glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate which are the precursors of the F-1,6-P were not oxidized. 3-Phospho-D-glycerate** and phosphoenol-pyruvate** were tested as the intermediates following F-1,6-P in the pathway of glycolysis. Neither of them were oxidized or scarcely oxidized.

2. Oxidation of Substrates by the "Glucose Cell" and the "Succinate Cell"—*Rhizobium* cells cultured using glucose as principal carbon source could readily oxidize glucose, whereas symbiotic cells could not oxidize glucose. Succinate and α-keto-glutarate were readily oxidized, but oxidations of pyruvate and fumarate were rather slow.

Among the intermediates of glycolysis tested, F-1,6-P was oxidized, but G-6-P, F-6-P and 3-P-Glyc. were oxidized very weakly suggesting the difficulty of penetration of these substances into the cells.

Rhizobium cells cultured on succinate showed rapid oxidation of succinate and fumarate. These cells had the ability to oxidize glucose and intermediates of glycolysis but the oxidizing power of glucose was rather weak compared with the "glucose cell". Since these cells were originally cultured on yeast-mannitol agar, it was reasonable that the oxidizing power for hexose had not been lost completely. Oxidation of G-6-P and F-6-P by the "succinate cell" was more rapid than that of F-1,6-P.

DISCUSSION

All of the *Rhizobium* cells grown in the three different media readily oxidized the organic acids which are the members of the Krebs cycle. This fact suggests the probable existence of this cycle in *Rhizobium.*

Glucose was not oxidized by the symbiotic cells, and only slowly by the "succinate cell", whereas the cells cultured on glucose oxidized glucose well. These results suggest that oxidation of glucose in *Rhizobium* is adaptive, and that glucose is not utilized by the symbiotic organism in the root nodules. Adaptation of *Rhizobium* to fructose or sucrose was not studied in this experiment. But it is known that *Rhizobium* can grow on these sugars as a sole carbon source in extranodular culture, and yet the symbiotic cell did not oxidize these sugars, therefore it is concluded that neither of them are supplied from the host plant to the bacteria in the nodules.

The lack of the ability to oxidize various substances may be considered as due either to the lack of the proper enzyme or to the difficulty of the penetration through the cell membrane. Whichever the case we can draw the same conclusion about the substrate of the oxidation supplied to the bacteria in the nodules.

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* Purity was confirmed by paper chromatography.

** Kindly supplied by Mr. Mori of this Department which he prepared from yeast.

Among the phosphate compound which are the intermediates of glycolysis, F-1,6-P was oxidized by both symbiotic and cultured cells, whereas its precursors and successors were not at all or only slightly utilized. A probable explanation is, that the penetration of these compounds into the cells was difficult except F-1,6-P.

From the results mentioned above it is concluded that the symbiotic bacteria receive from the host plant not sugars but organic acids of Krebs cycle. Succinate seems most likely substance among them.

Respiratory quotient of the root nodules as a whole was measured by one of the authors (*9*), to be between 1.01-1.17 which averaged 1.06 for soybean nodules and between 1.07-1.20 the average of which was 1.14 for hairy vetch nodules. They were higher than 1.00 for the oxidation of sugar, but not as high as for the oxidation of organic acids, ranging from 1.14 for succinic acid up to 1.60 for oxaloacetic acid. The respiration of the nodules is the sum of the respiration of the host cells and the symbiotic bacteria, so that it is not unlikely that the respiratory quotient of the nodules as a whole is close to that of sugars, even when the bacteria utilize only organic acids.

In this experiment only one strain of *Rhizobium* and one host plant species was studied. But there are some evidences in the literature supporting author's view. Thorn and Burris (*10*) compared the respiration of symbiotic cells and cultured cells (on yeast extract agar without the addition of other carbon source) of soybean, cowpea and pea bacteria. Their data on substrate oxidation was shown as the percentage of the rate of glucose oxidation. Thus the endogenous respiration of symbiotic cell of soybean and cowpea bacteria amounted to about 90 per cent of the respiration in the presence of glucose whereas the respiration with succinate reached 300 per cent. This suggests that glucose was not utilized at all. Their data with the symbiotic cells from pea and vetch nodules (same strain), endogenous and succinate respiration were about 50 and 160 per cent of the glucose respiration respectively. This suggests that glucose was oxidized by this strain but not so readily as succinate. We have occasionally observed that symbiotic vetch bacteria did not oxidize glucose (*6*). Therefore major substrate for respiration of symbiotic bacteria in the nodules may not be carbohydrate but organic acids, although some variations in respect of bacterial strain and host species might exist.

Recently Mumford *et al.* (*11*) obtained a mutant strain of *Azotobacter* which was unable to fix molecular nitrogen using glucose, sucrose or glycerol as substrate, except when organic acids such as lactate, pyruvate, malate, oxaloacetate and aconitate were supplied. This strain could grow using glucose when urea was supplied. They suggested that in this mutant strain the generation from glucose of some substrates required for the nitrogen fixing system was blocked. It seems that a similarity exists between this *Azotobacter* mutant strain and the symbiotic cell of *Rhizobium* in that the both bacteria utilize organic acids of Krebs cycle rather than glucose or sucrose for their nitrogen fixation.

SUMMARY

Three different cells of *Rhizobium japonicum*, *viz*. the cells grown on glucose, the cells grown on succinate, and the cells taken from soybean nodules, were compared in respect with the ability to oxidize substrates, including carbohydrates, the intermediates of glycolysis and the organic acids of Krebs cycle.

Organic acids were readily oxidized by all the three types of the cells. Among the hexose and triose-phosphates, only fructose-1,6-diphosphate was oxidized readily. Glucose was oxidized readily only by cells cultured on glucose, and slowly by the cells cultured on succinate, but not by the symbiotic cells. The symbiotic cells did not oxidize any of glucose, fructose, sucrose and mannitol. These facts suggest that the energy sources of *Rhizobium* in symbiotic state are organic acids but not carbohydrates, although it is possible that fructose-1,6-diphosphate is also used.

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A SIMPLE PREPARATIVE METHOD FOR RIBONUCLEOSIDE-2′,3′-CYCLIC PHOSPHATES

Despite a number of methods so far proposed (*1–6*), the preparation of cyclic nucleotides still requires skillful experimentation or expensive reagents. The purpose of this communication is to describe a simple and convenient method for the preparation of ribonucleoside-2′, 3′-cyclic phosphates directly from yeast ribonucleic acid (RNA) by the action of liquid ammonia. This method is also recommendable in view of an excellent yield of the cyclic nucleotides (about 80 per cent).

Two hundred milligrams of dried yeast RNA suspended in 1 ml. of formamide were well mixed with 5 ml. of liquid ammonia in a sealed tube, and the mixture was heated for 3 hours in a boiling water bath. Practically no inorganic phosphorus was liberated during the reaction. After completely removing ammonia from the reaction mixture, the products in the residual formamide solution were precipitated by adding 20 ml. of dried acetone and 30 ml. of dried ether. The precipitate thus obtained was a mixture of cyclic ribonucleotides containing a small amount of by-products. The separation of the cyclic nucleotides was easily effected by subjecting the formamide solution to paper chromatography in ammonia atmosphere using 70 per cent (by volume) aqueous isopropanol as solvent (*1*). Three main spots corresponding to cyclic adenylic acid (spot a), a mixture of cyclic pyrimidine nucleotides (spot b), and cyclic guanylic acid (spot c), together with two faint spots probably due to 2′- or 3′-nucleotides, were detected on the chromatogram.

These spots were separately eluted with water and their identity was confirmed as follows:

1. Paper chromatographic analysis of the base component in each eluate by the method of Smith *et al.* (*7*) gave relevant results.

2. While the eluate from spot b was completely digested by pancreatic ribonuclease to give ordinary pyrimidine nucleotides (3′-nucleotides) (*8*), the same treatment failed to alter the R_f values of the other spots in two different solvent systems.

3. The eluates from spots a, b and c were converted to corresponding ordinary mononucleotides (2′- and 3′-nucleotides) by the hydrolysis with 0.1 *N* HCl for 4 hours at room temperature (*9*) or with *N* NaOH for 1 hour at 100°. In the case of adenosine and guanosine cyclic phosphates (spots a and c, respectively), it was possible to separate the hydrolysis products into 2′- and 3′-nucleotides by paper chromatography using a mixture of saturated $(NH_4)_2SO_4$, *M* sodium acetate, and isopropanol as solvent (*10*).

4. No inorganic phosphorus was released from the ammonia degradation product of RNA by seminal phosphomonoesterase (*9*), unless the sample was

pretreated with 0.1 *N* HCl as described above.

The yield of total cyclic nucleotides estimated from the absorbancy at 260 mμ was 76 to 82 per cent of the total absorbancy of the product obtained after heating the RNA for 1 to 15 hours with liquid ammonia.

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Vol. 47 **No. 3**

CONTENTS

PAGE

SHIIO, Isamu. Bacterial formation of glutamic acid from acetic acid. I. Glutamate formation and its related enzymes in *Brevibacterium flavum*, No. 2247 273

MANO, Yoshitake. Studies on enzymatic synthesis of cocarboxylase in animal tissue. III. Purification and properties of thiaminokinase from rat liver.......................... 283

TITANI, Koiti, MINAKAMI, Shigeki and MITSUI, Hiromi. Cytochromes of *Pseudomonas-P.* Their formation and localization on cellular components............ 290

KATAGIRI, Hideo, YAMADA, Hideaki and IMAI, Kazutami. On the transphosphorylation reactions catalyzed by glucose-1-phosphate phosphotransferase of *Escherichia coli* 303

SUGAE, Kin-ichi and Honda, Yoshihide. Studies on bacterial amylase. III. On the amino-terminal peptide of bacterial amylase 307

OKUMURA, Nikichi, OTSUKI, Saburo and KAMEYAMA, Akira. Studies on free amino acids in human brain 315

MATSUSHIMA, Taijiro, IKENAKA, Tokuji and AKABORI, Shiro. Cysteic acid peptides from oxidized Taka-amylase A 321

HIGASHI, Taneaki. Physiological study on the oxygen- and nitrate-respiration of *Pseudomonas aeruginosa* 326

HORIE, Shigeo and TERADA, Shin-ichi. Studies on the distribution and phosphate-turnover of the acid-soluble phosphorus compounds in various normal and neoplastic tissues of rats. I. A semi-micro gradient elution chromatography with dowex 1 formate and identification of some phosphate esters on the chromatogram 335

MIZUSHIMA, Shôji and ARIMA, Kei. Mechanism of cyanide resistance in *Achromobacter*. I. Adaptive formation of cyanide resistant respiratory system in growing cells 351

TANAKA, Shozo, HATANO, Hiroyuki and GANNO, Shigetake. Actions of radiations on enzymes. III. The inactivating mechanism of sulfhydryl enzymes by γ-irradiation 361

HOSOYA, Toichiro. Turnip peroxidase. I. Purification and physicochemical properties of multiple components in turnip peroxidase 369

TAKEMORI, Shigeki. Studies on cytochrome A. V. Properties of copper in purified cytochrome A 382

TUZIMURA, Katura and MEGURO, Hiroshi. Respiration substrate of *Rhizobium* in the nodules 391

LETTERS TO THE EDITORS

TANAKA, Kentaro. A simple preparative method for ribonucleoside-2′,3′-cyclic phosphates 398